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Purification and Properties of *Cypridina* Luciferase[1]

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The light-emitting reaction accompanying the aerobic oxidation of the substrate, luciferin, by the enzyme, luciferase, extracted from the small ostracod crustacean, *Cypridina hilgendorfii*, has been studied from various points of view over a period of many years (Harvey, '17, '52; Tsuji, Chase and Harvey, '55). Among the systems known or presumed to occur among the many different types of luminescent organisms, the *Cypridina* system has appeared to be one of the simplest inasmuch as the evidence has indicated that, apart from water and molecular oxygen, no diffusible factors or enzymes in addition to the luciferin and luciferase, e.g., such factors as are associated with various other luminescent systems (cf. Johnson, '55; McElroy and Glass, '61) are involved in this instance.

Progress in the isolation of the essential components of the *Cypridina* system, however, has been fraught with numerous difficulties. Only recently has pure luciferin been successfully crystallized from extracts of dried organisms (Shimomura, Goto and Hirata, '57) as well as from extracts of initially live organisms preserved in dry ice (Haneda, Johnson et al., '61), and a tentative structure of the molecule arrived at (Hirata, Shimomura and Eguchi, '59; Shimomura, '60). Pure luciferin prepared from different specimens at different times has a constant light-emitting potency when tested with luciferase under standardized conditions. The ratio of this potency to that of a corresponding weight of the starting material, however, depends on the relative amount of luciferin in the specimens, and this amount has been found to vary widely in organisms collected at different seasons of the year, as well as with subsequent treatment of the specimens. Thus the increase in light-emitting potency as a result of purification ranges from an estimated 5000 to 100,000 times that of the starting material, on a weight basis.

Partially purified luciferase, with a potency of some 150 times that of the crude extract from dried organisms, has been obtained through a procedure involving chromatographing on a calcium phosphate gel and fractionating with acetone and ammonium sulphate (McElroy and Chase, '51). The present study has resulted in essentially complete purification of *Cypridina* luciferase, both from dried organisms and from specimens preserved in dry ice, with an estimated potency of about 4000 times that of the live organisms. Experience with specimens preserved in either manner, and collected at various seasons, has indicated that, unlike luciferin, the luciferase content of the starting material is usually almost the same. Some of the important characteristics of the purified enzyme, including molecular weight and the influence of various agents on catalytic activity, have been determined as described herein.

MATERIALS AND METHODS

Raw material

The raw material used in this study included specimens collected from various localities along the shores of Japan at different seasons of the year, and kept for various lengths of time up to as much as 10 years in the dry state, or for several weeks in dry ice.

[1] This study has been aided in part by Contract Nonr-1353(00) between the Office of Naval Research and Princeton University, by National Science Foundation grants G-4578 and G-12857, and by the Eugene Higgins Fund allocated to Princeton University. Publication in whole or in part by or for any purpose of the United States Government is permitted.

Measurement of luciferase activity

Luciferase activity was assayed at room temperature with the aid of a photomultiplier-amplifier apparatus which could measure either instantaneous luminescence intensity or the integrated total light as a function of time. The specimen holder was shielded from extraneous light so that 10 ml of an appropriate dilution of luciferase, usually in 0.1 M sodium phosphate buffer in 0.2 M sodium chloride pH 6.9–7.0, could be quickly added by volumetric pipette to 0.1 ml of a methanolic solution of luciferin containing 100 to 120 μg of luciferin per ml. The final luciferin concentration, 1.0 to 1.2 μg/ml, was saturating for a fairly wide range of luciferase concentrations and, except where noted otherwise, a saturating concentration that gave a luminescent reaction zero order with respect to substrate concentration was employed. Enzyme activity was conveniently measured on a Sanborn recorder in terms of the slope of the line relating the practically linear increase in total light during the first 15 to 20 seconds, or longer, after adding the enzyme. An alternative, equally convenient method, taking advantage of the zero order kinetics, consisted simply in measuring the level of instantaneous luminescence intensity for a brief period after adding the enzyme. One "luciferase unit" ("LsU") was arbitrarily defined as the activity required for an increase in total light of 0.5 × 1 smallest division (full scale = 50 smallest divisions) per second on the recorder, or alternatively, as the activity required for a steady, instantaneous intensity of 0.5 smallest division, with the amplifier gain set at 100×10^{-5} coulombes, or 100×10^{-6} amperes, respectively.

The range of activities that could be satisfactorily measured under these conditions extended from about 0.5 to 4 LsU, with an accuracy of ±5% in repeated experiments. In different batches of raw material the luciferase activity amounted at best to 20 LsU/mg dried specimens and 5 LsU/mg wet weight of fresh specimens. The most highly purified product had an activity of 20,000 LsU/mg protein.

Purification procedures

Two methods of initial purification, eac with certain time- and labor-saving adva tages over those hitherto employed f this enzyme, were used: (1), precipitatic of the active luciferase from an aqueou extract of ground *Cypridina* by addin Rivanol (= Acrinol = Ethodin = 6,9-d amino-2-ethoxyacridine lactate) to a fin concentration of 0.3% at pH 5.5 and at temperature below 5°C, followed by r covery in 3% ammonium sulphate at p 8.0 to 8.5; and (2), adsorption of th active luciferase in the aqueous extract c DEAE cellulose, followed by elution ar repeated chromatography on a DEA column.

In the first of these methods, 97% the original luciferase activity is containe in the yellowish-brown Rivanol precipita collected by filtration with filtercel c paper in a Buchner funnel. The filtra contains dark, coffee-colored impuritie which, according to earlier procedures, a eliminated by dialysis. The caked precip tate contains considerable amounts of pr tein impurities. About 75% of the lucife ase is recovered in the dilute ammoniu sulphate, which takes up a small amou of Rivanol also, but relatively little of th protein impurities. Addition of a sma amount of acidic clay (Wako Chemic Co., Osaka, Japan), and filtration throug paper on a Buchner yields a clear, pract cally colorless filtrate containing lucife ase in a purity of 20 to 30% by weight protein.

Despite the tempting simplicity of th Rivanol procedure, it was found that an method involving precipitation of the en zyme resulted in considerable loss of a tivity. The procedure using DEAE at th start, therefore, was preferable, and gav the purest preparations in the best yield

For illustration, the details of a typic experiment are as follows.

Five hundred grams of dry *Cypridin* were ground in a mortar and extracte with 5.5 liters of distilled water with slo stirring in the cold room at 4°C overnigh then filtered through a large Buchner wit the aid of filtercel. The dark colored fi trate was diluted with distilled water unt the chloride concentration became le

han 0.07 molar as judged by titration of mall aliquots with 0.1 N $AgNO_3$. The pH f the solution, now about 10 liters in olume, was adjusted to 7.0 by adding ilute acetic acid. One hundred grams of)EAE were stirred into the solution and eft overnight in the cold room. More han 98% of the luciferase activity was dsorbed on the DEAE which was filtered ut the next day; the dark colored filtrate vas discarded. The DEAE cake was mixed vith 1.5 liters of buffered salt solution onsisting of 0.01 M sodium phosphate nd 0.1 M sodium chloride, pH 7.0, and he resulting slush was transferred to a ubular glass column 50 cm long and 6 cm d. After most of the salt solution had rained out, the luciferase was eluted by neans of a similar buffered salt solution xcept that the sodium chloride concen- ration was 0.3 M instead of 0.1 M. The irst 600 ml of eluate contained about 0% of the original luciferase activity.

This first eluate was further purified y chromatographing 5 successive times n a DEAE column, lowering the pH of he solution and reducing the quantity f DEAE each time, ending with only gm of DEAE in a small column, and uffer of pH 6.9. For the first run, 30 gm f DEAE were added to distilled water nd the pH of the mixture was adjusted o 7.4 by adding Na_2HPO_4. After settling, he free liquid was decanted and a solu- ion of 0.01 M sodium phosphate and 0.05 M sodium chloride, pH 7.4, was dded. The mixture was transferred to a lass column and packed under a nitro- gen pressure of 10 lbs./in^2. Before adding o the column, the original 600 ml eluate f luciferase was dialyzed against 0.01 M odium phosphate buffer, pH 7.4. The ialyzed luciferase became adsorbed at the op of the column. Elution was accom- lished by the gradient dilution method, with gradual change in NaCl concentra- ion through continual addition of 0.01 M uffer containing 0.5 M NaCl solution in ne flask, to 1.3 liters of the correspond- ng buffer solution containing 0.05 M NaCl n the first flask. The eluate of the first un was collected in 12-ml aliquots by neans of an automatic fraction collector. Both luciferase activity and absorption at 278 mμ were measured on each aliquot. In successive runs, the aliquot volume was reduced, ending with 1-ml portions in the 5th run. In each run, only those fractions were saved that had the highest ratios of luciferase activity to ultraviolet absorption, aggregating up to 75% of the total activity placed on the column. Thus, after the 5th successive chromatograph, the yield amounted to approximately 20% of the luciferase in the starting material of dried organisms.

RESULTS OF EXPERIMENTS

Activity versus UV absorption

Figure 1 shows the quantitative relation between luciferase activity and ultraviolet absorption at 278 mμ, of sequential fractions collected from a sample of solution, in testing for purity, after the 5th chromatograph. The correspondence between activity and UV adsorption is sufficiently close to conclude that the preparation is very highly purified. The pooled fractions numbering 14 to 21 inclusive, representing most of the total activity in about 10 ml, gave an activity of about 20,000 LsU per mg of protein, which amounted to 6.6 mg/ml precipitable by trichloracetic acid.

Absolute luciferase concentration

The above data furnish a means of estimating the absolute amount of luciferase both in the living organisms and in raw material of unknown content, provided the influence of possible impurities inhibitory to catalytic activity can be ruled out, as by sufficient dilution. Thus, 0.005 μg of luciferase per ml give one luciferase unit under the conditions described. A different light measuring apparatus could be calibrated to corresponding units on the basis that one μg of pure luciferin, with excess luciferase, emits a total light of 250 smallest divisions on the scale used in the present work. Hence 1 LsU would emit 1/500 of this total per second for the first few seconds under the appropriately standardized conditions.

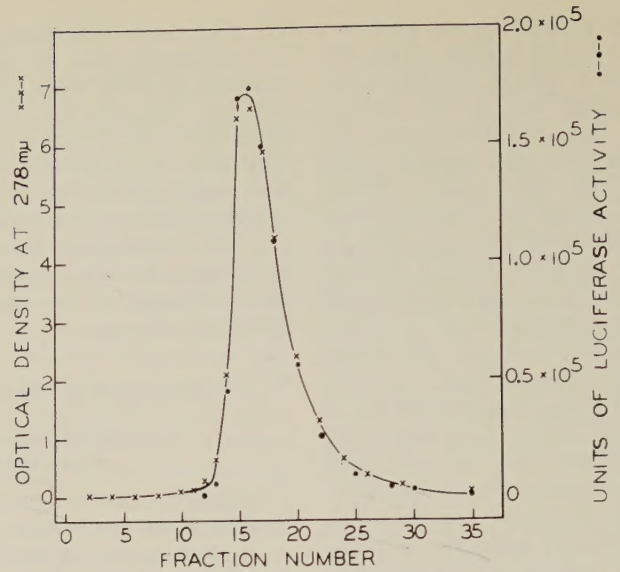

Fig. 1 Activity and UV absorption of luciferase fractions at pH 7.0 after chromatographing 5 times on DEAE as described in the text.

With respect to the amount of luciferase in individual specimens of *Cypridina*, the average weight of a dried organism, according to figures given by Harvey ('52), is about 1 mg, and since 500 gm of dried organisms contain 10,000,000 units of luciferase at 20 units per μg, it follows that 1 dried organism contains 1 μg of luciferase. The living organism would contain the same amount, provided none of the enzyme were lost in the process of collecting and drying. The fact that freshly collected specimens yield about the same amount of luciferase as the dried specimens, allowing for 75% weight loss on drying, indicates that no significant loss of luciferase occurs during the drying process, but appreciable loss might be expected during collecting because of the unavoidable stimulation to secrete luminescent slime through handling of the animals. In terms of molar concentration, it may be concluded that the average *Cypridina* specimen has 2×10^{-11} moles of luciferase, with a molecular weight of about 50,000 (*vide infra*), or about one molecule of luciferase per 100 molecules of luciferin, assuming a maximum amount of 0.1% luciferin with a molecular weight close to 500 (cf. Haneda, Johnson et al., '61).

Absorption spectrum

The ultraviolet and visible absorpti spectrum of luciferase in phosphate buff at neutral pH is represented in figure A sharp maximum occurs at 277 m which is within the range of 275–280 n reported for the fractions with highest a tivity obtained by McElroy and Cha ('51), as well as within the 275–278 n reported by Osborn and Chase ('54). T character of the absorption curve is co mon to various proteins with no maxi in the visible, and is indicative of the a sence of an organic prosthetic group.

Ultracentrifugal analysis

The data from two runs in a Spin analytical centrifuge are illustrated figures 3 and 4. The first run (fig. revealed a single peak and a sediment tion constant of 3.93 Svedberg units (S with no evidence of appreciable amoun of impurities having a higher S, but b cause of the uncertainty of the base li in this experiment, impurities having lower S value could not be readily d tected. A second run was therefore ma with the same material, after about o month and re-chromatographing on DEA and dialyzing. In this instance the sy

netic boundary method was used. The esults, illustrated in figure 4, indicate ne presence of a small amount, about %, of high molecular weight impurity hich was absent in the first run. It is reasonably likely that this material consists of impurity from the DEAE, or possibly from the cellophane tubing used for dialysis, that was not removed by preliminary centrifugation for a few minutes at

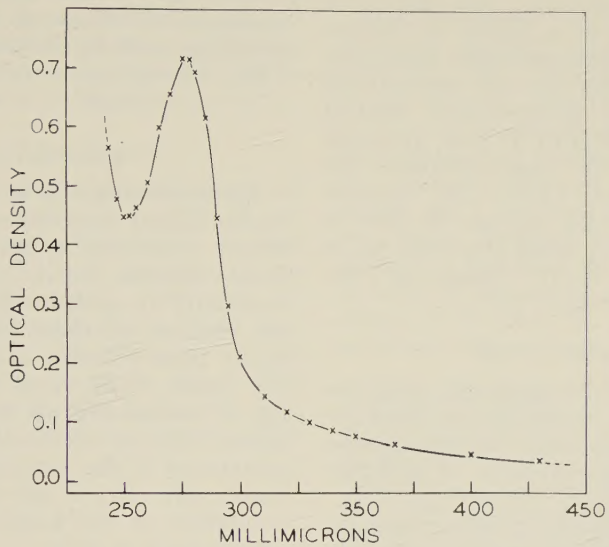

Fig. 2 Absorption spectrum of luciferase in 0.1 M sodium phosphate buffer, pH 7.0. At wavelengths longer than the 430 mμ shown in the figure, absorption decreases progressively to practically nil at 600 mμ.

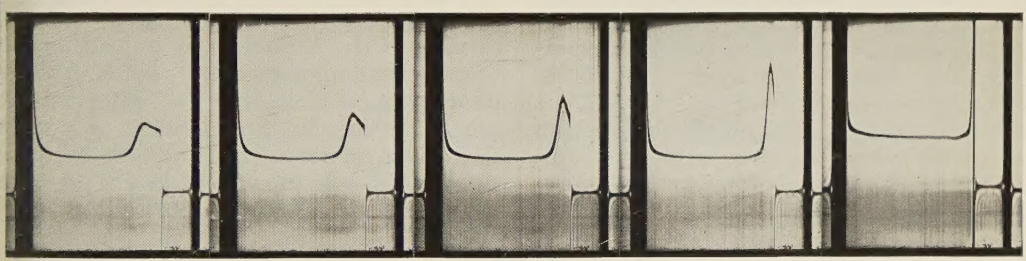

Fig. 3 Sedimentation of luciferase at 59,780 rpm in 0.1 M sodium phosphate buffer, pH 7.0, with .2 M NaCl, at 1.5°C. From right to left, photographs were taken at 4, 16, 28, 40 and 52 min., espectively.

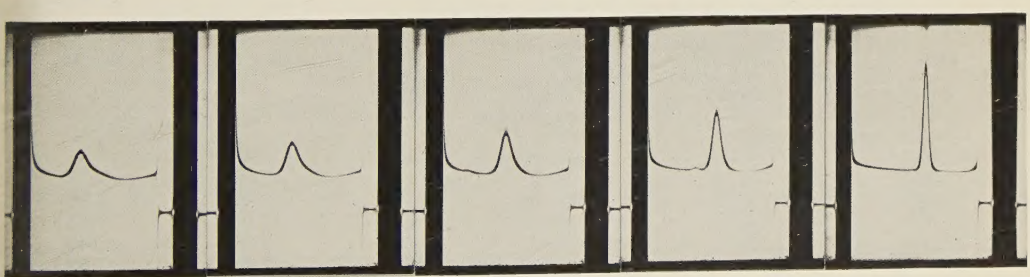

Fig. 4 Sedimentation of luciferase in 0.04 M sodium phosphate buffer, pH 7.0, and 0.1 M sodium loride, by the synthetic boundary method, at 3.6°C; 59,780 rpm, 5.9 mg protein/ml. From right left, the photographs were taken at 4, 16, 28, 40 and 52 min., respectively.

3000 rpm in an ordinary centrifuge. The data of figure 4 lead to a value of 4.3×10^{-13} as the sedimentation constant.

Diffusion constant

The diffusion constant was measured in a 2 ml cuvette of a Model 38 Perkin-Elmer boundary electrophoresis apparatus at 20°C, with results that are summarized in table 1. Using the zero time method of Lundgren and Ward ('51), an averaged value of 7.65×10^{-7} was calculated according to area, and 7.625×10^{-7} according to inflection. The average of these is 7.638×10^{-7}, which when corrected to the viscosity of water at 20°C comes to 7.88, or approximately 7.9, $\times 10^{-7}$.

Molecular weight

On the basis of the foregoing data, the molecular weight of luciferase falls between 48,500 and 53,000. In this calculation a partial specific volume of 0.75 ml/gm was assumed.

Values for the sedimentation constant, diffusion constant, and molecular weight of luciferase in less highly purified preparations have been determined earlier on the basis of some ingenious experiments by Chase and collaborators (Chase, '55; Fedden and Chase, '59; Chase and Langridge, '60), whose results are in remarkably good agreement with the above. Thus with a method utilizing the fixed partition cell in the ultracentrifuge and determining luciferase concentration by light-emitting activity, S_{20} was found to be $(5.3 \pm 0.2) \times 10^{-13}$ sec., which in conjuncti with a value of $D = 7.4 \times 10^{-7}$ for t diffusion constant obtained from rate passage through a porous disk accordi to the method of Northrop and Ans ('29), the concentration of lucifera again being measured in terms of ligl emitting activity, Chase and Langridl ('60) arrived at a molecular weight $(7.0 \pm 0.5) \times 10^4$.

Electrophoresis

Electrophoresis was studied with t Perkin-Elmer apparatus and also on pap Since, according to our experience, luc erase adsorbs fairly strongly on pap especially at acidities greater than pH the former of these methods no dou yields more reliable values of the isoele tric point. The data are illustrated fig. 5, indicating an isoelectric point about 4.35 by extrapolation of the cur pertaining to the amount of migration higher pH. Below pH 4.5, very rapid i activation of the highly purified enzyn occurs even at 0°C.

The unusually acid isoelectric point 3.28 reported by Weir, Tsuji and Cha ('55), on the basis of paper electrophor sis, is possibly to be interpreted as d in part to the influence of impurities. the present experiments, paper electr phoresis gave a value of 4.7 after corre ing according to the mobility of dextr (15 hours on Schleicher and Schuell pap no. 2043-B, one inch wide, at 4°C, 4 volt cm, using as solvent 0.04 M sodium pho

TABLE 1

Data on the diffusion of luciferase in 0.04 M sodium phosphate buffer, pH 7.0, and 0.1 M NaCl, at 20 ± 0.01°C

Time	H_m (Maximum height of the curve)	A (Area under the curve)	$\chi\mu$ (Half width of curve at $H_m/\sqrt{e}$ Height	$D_A \times 10^7$	$D_I \times 10^7$
seconds	*cm*	*cm²*			
3,600	4.12	1.08	0.10	7.38	
7,200	3.32	1.09	0.13	7.63	7.77
14,400	2.56	1.10	0.17	8.02	7.98
32,400	1.86	1.12	0.23	7.95	7.35
79,200	1.30	1.13	0.35	7.27	7.40
Average				7.65	7.625

$D_A = A^2/[4\pi t_o'(H^2)]$.
$D_I = \mu^2/2t_o''$.

hate or potassium bi-phthalate buffer, ither buffer containing 0.2 M sodium hloride).

Electrophoresis with a higher concenration of protein, 0.8% instead of 0.2%, evealed only a single peak (fig. 6) with very slight bulge at the origin; the latter ould be due to an impurity comprising ess than 5% of the total protein, or it night be only the delta band.

Analysis for metals

Emission spectral analysis of 16 mg of uciferase protein and of a portion of the .001 M sodium phosphate buffer against vhich it has been dialyzed, revealed traces f various metallic ions, only two of vhich, viz., copper and silicon, were conained in a higher concentration in the nzyme preparation than in the buffer (table 2). On a molecular basis, the ratio f this amount of copper to protein is less than 1 in 10, so it can scarcely be considered significant in the activity of the enzyme. An essential role of silicon seems extremely unlikely.

Activators and inhibitors

Data obtained earlier with very considerably purified luciferase (Chase and Lederman, '61) indicate that the activity of this enzyme goes through a maximum at a low concentration of phosphate buffer. Dialysis of the present enzyme material against 0.001 M sodium phosphate buffer, pH 7, followed by a 1:100,000 dilution with distilled water, reduced the activity to less than 5% of the maximum. Activity of this practically salt-free preparation could then be restored to various extents by the addition of different cations

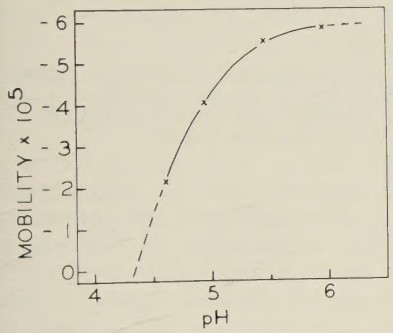

Fig. 5 Electrophoretic mobility of about 0.2% uciferase solution as a function of pH in 0.04 M odium phosphate buffer and 0.1 M sodium chloride; voltage 70 v, time 45 min., ice bath temperature.

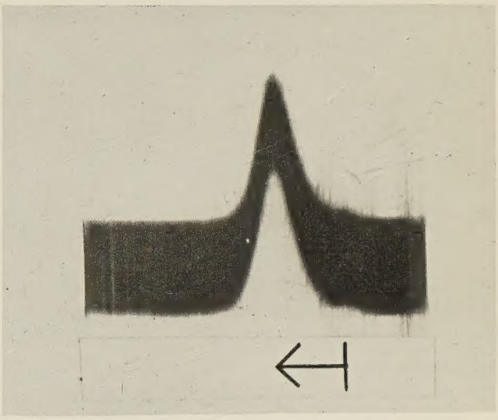

Fig. 6 Electrophoretic pattern of luciferase at a concentration of 0.8% protein in the same buffered salt solution used for figure 5, at pH 5.5; voltage 60 v, time one hour, ice bath temperature. Ascending boundary.

TABLE 2

Analysis by emission spectroscopy[1] for metal content of 16 mg of luciferase protein in 3 ml of buffer, and of 3 ml of the buffer used in dialyzing the enzyme solution

Metal	Parts per million	
	Enzyme	Buffer
Calcium	Not detected	4
Copper	2	Not detected
Iron	Not detected	2
Magnesium	1	2
Sodium	30	120
Silicon	30	8
Zinc	8	40
Manganese	Not detected	4
Cobalt	Not detected	Not detected

[1] Schwarzkoph Microanalytical Laboratory.

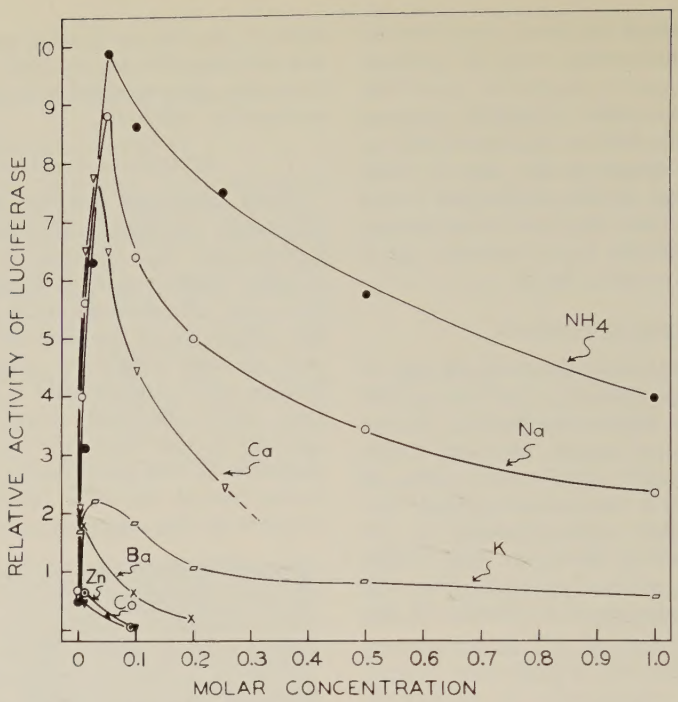

Fig. 7 Activity of luciferase at 25° C as a function of concentration of different cations. The pH of the solutions, just after measuring enzyme activity, ranged between 5.9 and 6.8. In only two instances, viz., at 0.1 M $CoCl_2$ and 0.1 M $ZnCl_2$, respectively, an initial adjustment of pH was made, in each instance by addition of a trace of NaOH.

as shown in figure 7. Among the ions included, ammonium, sodium, and calcium are the most effective activators, whereas potassium, zinc, and cobalt, respectively, are relatively ineffective.

Heavy metals, as expected, inhibited at rather low concentrations, e.g., 6×10^{-5} M $AgNO_3$ caused a 50% inhibition, and 5×10^{-4} M $HgCl_2$ a 30% inhibition of activity at room temperature in 0.1 M sodium acetate buffer, pH 6.5. Parachloromercuribenzoic acid caused only a 10% inhibition, even at a concentration of half-saturation, indicating the absence of functionally active SH groups on the enzyme. Mild reducing agents, such as glutathione, cysteine, and ascorbic acid, had almost no effect on activity. Strong reducing agents, such as $Na_2S_2O_4$, exerted inhibitory effects on the luminescent reaction only when added in concentrations sufficient to reduce the amount of dissolved oxygen.

The influence of chelating or complexing agents, alone and in conjunction with various metallic ions, is summarized in table 3. Although only one concentration of EDTA is included in this table, it should be mentioned that the effects of this substance showed a peculiar relation between concentration and amount of inhibition. Thus, 25×10^{-7} M had virtually no effect on luciferase activity. Five times this concentration caused a 13% inhibition, and 10 times the same concentration, i.e., 25×10^{-6} M, resulted in an 84% inhibition. Further increases in concentration, even to 5×10^{-3} M, increased the inhibition only to about 90%. On dialyzing two days against several changes of 0.01 M phosphate buffer, pH 7.0, full activity was restored.

Referring to table 3, several noteworthy relations are apparent. First, the effects of the strongly inhibitory concentrations of EDTA are fully, or practically fully, counteracted by small concentrations of zinc, manganese, cobalt, or calcium. In the absence of EDTA, only copper is strongly inhibitory at the metal concen-

tration involved, and in the presence of EDTA the combined effect of the two is only a slightly less inhibition than that due to either one by itself. Magnesium and barium, which by themselves are not inhibitory, have only a slight effect on the amount of inhibition by EDTA, with which they chelate weakly. These data are evidently to be interpreted on the basis of the relative combining strength of EDTA with the metallic ions and the combining strength of each with luciferase. There is no clear indication that any metallic ion is functionally significant in the catalytic activity of the enzyme. It is not surprising, therefore, to find that cyanide by itself does not inhibit and that spectrographic analysis, mentioned above, revealed no significant amount of metal in the enzyme.

The inhibition caused by the combined action of either Zn or Co with *o*-phenanthroline or 8-hydroxyquinoline is peculiar in being very much greater than that caused by any one of these agents alone. While the mechanism of this effect is uncertain, it is probably associated with the fact that each of these metals forms a strong complex with each of these organic substances. The resulting complex constitutes a different and more strongly inhibitory agent than its separate components.

Calcium has a small activating effect by itself, and a strong re-activating effect in the presence of the other inhibitors, with the exception of KCNS. This last-mentioned substance, however, reduces the total amount of emitted light as well as influencing the activity of the enzyme. Its action is to be attributed in part to a quenching effect, i.e., to a reduction in the efficiency of light emission, as noted earlier by Anderson ('37).

Activity versus pH

At room temperature, with a favorable salt composition of 0.05 M NaCl and 0.005 M sodium phosphate buffer, the pH-activity curve illustrated in figure 8 was obtained. The maximum is at about pH 7.5, which is essentially the same as that found by Chase ('48) with 0.01 M sodium phosphate buffer. As Chase showed, the position of the maximum and the shape of the curve, however, are subject to variation with the cationic composition, and in general, they may be expected to vary to a greater or less extent under the influence of other factors including temperature and hydrostatic pressure (Johnson, Eyring and Polissar, '54).

The stability of luciferase at 25°C,, when exposed briefly to different pH values over a wide range, which necessitated the use of more than one buffer system, is also shown in figure 8.

TABLE 3

Inhibition (per cent) of luciferase by metallic ions and chelating or complexing agents

Separately and in combination, at 25°C, in 0.03 M sodium phosphate buffer and 0.06 M sodium chloride, pH 7.0, except for citrate solution without the phosphate. Cation concentration 0.001 M in each case. All metallic salts were chlorides except those of copper and magnesium which were sulphates.

Complexing or other substance	No added metallic ion	Cu++	Zn++	Mn++	Co++	Mg++	Ba++	Ca++
None	0	93	15	10	5	0	0	−10
EDTA 0.0005 M	90	80	0	5	5	70	85	0
KCNS 0.002 M	82	100	82	82	82	82	82	82
o-Phenanthroline 0.001 M	10	72	100	73	74	15	15	5
KCN 0.001 M	0	100	12	20	[1]	25	25	0
Na-diethyldithiocarbamate 0.001 M	0	100[2]	100[2]	35[2]	100[2]	13	13	−15
Na-citrate 0.05 M	60	82	50	10	60	50	50	0
8-Hydroxyquinoline 0.001 M	0	90[2]	64	20	95[2]	10	5	0

[1] No simple numerical figure can be given because of complex kinetics.
[2] Visible precipitation.

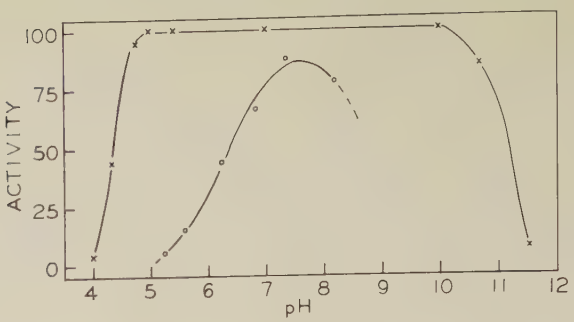

Fig. 8 Influence of pH on luciferase activity (circles) and stability (crosses) at 25°C. For the pH-activity curve, 0.005 M sodium phosphate buffer containing 0.05 M sodium chloride was used. For the stability tests, 5 μl of luciferase solution were first added to 0.5 ml of dilute (less than 0.01 M) buffer, and after 10 minutes 9.5 ml of 0.1 M sodium phosphate buffer containing 0.2 M sodium chloride at pH 7.0 were added and the activity determined. Stability at pH 4 and 4.35 was tested in potassium biphthalate; at pH 4.75 to 7 in sodium phosphate; and at pH 10 to 11.5 in sodium carbonate.

The Michaelis-Menten constant

Before the molecular weight of luciferin was established, the Michaelis-Menten constant at 22°C was computed as 1.24 $\times$ 10^{-6} moles/liter on the basis of data obtained with the incompletely purified system, using 300 as a reasonable value for the molecular weight of luciferin (Kauzmann, Chase and Brighham, '49). According to the empirical formula of pure luciferin the molecular weight is 469 (Hirata, Shimomura and Eguchi, '59), or approximately 470. Using this value in conjunction with data obtained in the present experiments (fig. 9) leads to 0.52 $\times$ 10^{-6} as the Michaelis-Menten constant at 25°C.

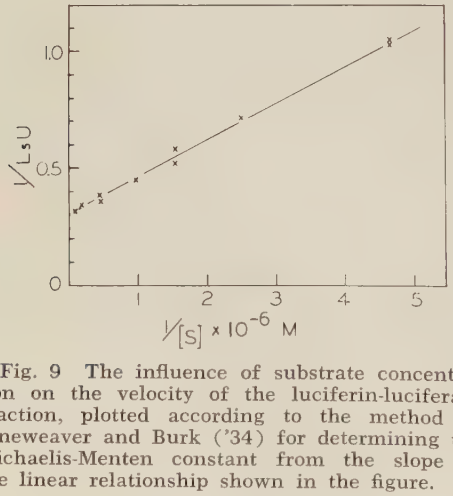

Fig. 9 The influence of substrate concentration on the velocity of the luciferin-luciferase reaction, plotted according to the method of Lineweaver and Burk ('34) for determining the Michaelis-Menten constant from the slope of the linear relationship shown in the figure.

SUMMARY

The enzyme luciferase, of the luminescent system of *Cypridina*, has been purified from aqueous extracts of both fresh and dried organisms by (1), repeated adsorption on and elution from DEAE cellulose columns, and by (2), the same procedure after initial precipitation with Rivanol then elution and filtration through acid clay. The essentially pure product has an activity about 4000 times that of living organisms.

Ultracentrifugal, electrophoretic, spectrographic and other data indicate that Cypridina luciferase has the following properties. It is a simple protein without a metallic or organic prosthetic group; it has a sedimentation constant of 4.3 $\times$ 10^{-13} a diffusion constant of 7.9 $\times$ 10^{-7} a molecular weight between 48,500 and 53,000, an isoelectric point of 4.35, a pH optimum at 7.5 in sodium phosphate buffer at room temperature, and it is rapidly inactivated at pH more acid than 4.7. Catalytic activity is uninhibited by 0.001 M KCN, Na-diethyldithiocarbamate, 8-hydroxyquinoline, and only very slightly by *o*-phenanthroline and half-saturated p-chloromercuribenzoic acid. It is strongly inhibited by 0.0005 M EDTA in a manner that is reversible by dialysis or by addition of 0.001 M Zn, Mn, Ca, or Co, but not Cu which is strongly inhibitory of itself, and not by the weakly chelated cations Mg or Ba. Activity practically disappears in nearly complete absence of salts, but is re

stored by adding small concentrations of NH_4, Na, or Ca, though only very slightly by K, Ba, Zn, or Co. Strong inhibitions result from the combined action of Zn or Co with o-phenanthroline or 8-hydroxyquinoline. The Michaelis-Menton constant is 0.52×10^{-6} under the conditions involved.

The absolute concentration of luciferase in living Cypridina is estimated to average about 1 μg, or 2×10^{-11} moles, per individual organism.

ACKNOWLEDGMENTS

The authors gratefully acknowledge their indebtedness to Dr. Y. Hirata of Nagoya University, for supplying more than 5 kg of dry Cypridina for the start of research on this problem at Nagasaki University; to Miss Michiko Ogawa for assistance in the preliminary experiments; to Dr. Y. Haneda and Dr. N. Sugiyama for cooperation in supplying raw material and laboratory facilities at the Tokyo Kyoiku University; and to Dr. Verne Schumaker and his assistants, Miss Barbara Marano and Miss Joan Rosenbloom, through whose cooperation the ultracentrifugal data and analyses were made possible.

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Purification and Molecular Weight of *Cypridina* Luciferase[1,2]

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The blue luminescence produced by the marine ostracod crustacean, *Cypridina hilgendorfii*, is due to the oxidation of luciferin by molecular oxygen, catalyzed by the enzyme luciferase. The light emitting reaction may be written as follows:

$$\text{luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{blue light} + \text{oxidized luciferin}$$

Although a great deal of knowledge exists concerning the chemistry of luciferin, comparatively little is known regarding some of the physical and chemical properties of luciferase.

Chase, Schryver and Stern ('48) have carried out free electrophoresis studies on dialyzed water extracts of *Cypridina* organisms and have found one stationary and two mobile components. Luciferase activity appeared to migrate with the component of lower mobility at pH 7.6. By means of paper electrophoresis, Weir, Tsuji and Chase ('55) have obtained an isoelectric point of 3.28 for luciferase. McElroy and Chase ('51) have purified luciferase 150 fold by fractional precipitation with acetone and $(NH_4)_2SO_4$, and subsequent adsorption and desorption from calcium phosphate gel. Using a purified fraction (fraction II) prepared by the preceding method, Chase ('55) has determined the molecular weight of luciferase to be about 35,000–39,000 from sedimentation-viscosity data. Subsequently, Fedden and Chase ('59) were able to obtain a diffusion constant of 7.4×10^{-7} cm^2/sec. by the porous disk method of Northrop and Anson ('29), from which the radius of the molecule was calculated by the Einstein equation relating radius to the diffusion constant. From a knowledge of the radius and assuming a specific gravity of 1.33 for luciferase, a molecular weight of 80,000 was obtained. On the other hand, when the diffusion constant was employed in conjunction with a sedimentation constant of 3.4×10^{-13} sec. determined earlier by Chase ('55), the molecular weight of luciferin was found to be about 45,000.

In order to prepare luciferase sufficiently pure for physical-chemical studies, and to clear up the molecular weight discrepancy, the present investigation was carried out. Luciferase was purified by fractional precipitation and by gradient elution chromatography. The purified luciferase was then used for the determination of molecular weight by the technique of sedimentation-viscosity.

MATERIALS AND METHODS

Luciferase was purified by a procedure combining a modification of the method of McElroy and Chase ('51) with chromatography on DEAE cellulose.[4] Due to the fact that a number of changes were made in the method of McElroy and Chase, the procedure is described in full below.

Crude extract of Cypridina hilgendorfii. Finely ground *Cypridina* organisms, prepared from the dried material, were extracted with distilled water in the ratio of 100 gm of powder to 800 ml of water. The extraction was carried out at 4°C for 18 hours with continuous stirring. After

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[2] Publication no. 91 of the Department of Biophysics.

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[4] Diethylaminoethyl cellulose (Carl Schleicher and Schuell Co., Keene, New Hampshire).

centrifuging for 20 minutes at approximately 750 × g, the supernatant containing the dissolved luciferase was dialyzed against cold, running tap water at approximately 10°C for 18 hours. The luciferase solution was then centrifuged at approximately 20,000 × g for 20 minutes to remove precipitated material that formed during the dialysis. The clear solution was then dried from the frozen state to give a light brown powder, which was stored at −15°C until used. The powder possessed a specific activity of 11 light units (arbitrary) /mg nitrogen compared to a specific activity of 1 light unit (arbitrary) /mg nitrogen for the starting material. Nitrogen was determined by the conventional micro-Kjeldahl technique.

Fractional precipitation with acetone and $(NH_4)_2SO_4$. A 1.5% water solution of the powder (w/v) was prepared and ice-cold acetone was slowly added to a final concentration of 30% (v/v) while the mixture was continually stirred in an ice-bath. All subsequent manipulations were carried out between 0–4°C. After standing overnight, the mixture was centrifuged at approximately 750 × g for 30 minutes and the precipitate discarded. To the supernatant 0.1 M phosphate buffer, pH 5.5, was added in the ratio of 8.0 ml of buffer for every 70 ml of supernatant. Cold acetone was then added to a concentration of 60% (v/v) and the mixture allowed to stand for an hour. The mixture was centrifuged at approximately 750 × g for 30 minutes and the supernatant discarded. The precipitate was then dissolved in the smallest volume (~ 100 ml) of 0.1 M phosphate buffer, pH 6.8, and solid $(NH_4)_2SO_4$ added to bring the solution to 40% saturation (based on solubility of $(NH_4)_2SO_4$ at 0°C, w/v). After half hour standing, the mixture was centrifuged at approximately 750 × g for 30 minutes and the precipitate discarded. To the supernatant, solid $(NH_4)_2SO_4$ was next added to raise the concentration to 60% saturation. After standing for one hour, the mixture was centrifuged at approximately 750 × g for 30 minutes and the supernatant discarded. The precipitate was redissolved in a small volume of 0.2 M phosphate buffer, pH 6.8, and dialyzed against repeated changes of distilled water to remove dissolved salt. After freeze-drying, the light-brown powder was stored at −15°C until used. The prepared material possessed a specific activity of 136 light units (arbitrary) /mg nitrogen compared to the dried organisms.

Purification on DEAE column. The DEAE column was prepared by adding a fine suspension of DEAE cellulose in 0.07 M phosphate buffer, pH 6.8, to a column possessing a coarse, sintered glass disk at the bottom. The DEAE cellulose, previously washed thoroughly with buffer, was poured in sections. After each addition of the suspension, the DEAE particles were allowed to settle by gravity. The DEAE was then carefully packed down with a flat ended glass rod and the excess buffer drained off to the top of the DEAE column before more suspension was added. Following equilibration with 0.07 M phosphate buffer, pH 6.8, the column had a dimension of 1.5 × 37.5 cm.

Eighty to 120 mg of the powder from above were dissolved in approximately 10 ml of 0.07 M phosphate buffer, pH 6.8, and dialyzed overnight against the buffer. After centrifugation at approximately 750 × g, the clear, brown solution was placed on the column. Gradient elution was carried out by allowing 300 ml of 0.80 M NaCl made up in 0.07 M phosphate buffer, pH 6.8, to flow from a spherical container (Florence flask, 300 ml capacity) into a cylinder (radius = 4.2 cm.) containing 390 ml of 0.05 M NaCl made up in 0.07 M phosphate buffer, pH 6.8. The cylinder was stirred with a magnetic stirrer, employing a teflon covered magnet. The containers were connected by a siphon arrangement that maintained the liquids at the same level at all times. The influent flowed from the cylinder into the column and the flow rate of the effluent was 0.9 ml/min. Six-milliliter fractions were collected and aliquots were assayed for activity with luciferin. Under these conditions, the concentration of NaCl increased almost linearly for 350 ml of effluent collected.[5] Luciferase was eluted at

[5] NaCl concentration determined conductometrically using LKB Conductivity Bridge, Type 3216B (Ivan Sorvall, Inc., Norwalk, Conn.).

a concentration close to 0.35 M NaCl. A considerable amount of dark-colored material, devoid of luciferase activity, remained adsorbed at the top of the column throughout the elution process. Fractions possessing the highest activities were combined, dialyzed against distilled water, and dried from the frozen state. A whitish powder was obtained which was re-chromatographed a second time. The final, dried power possessed a specific activity of 461 light units (arbitrary) /mg nitrogen when compared with the starting *Cypridina* material. The powder was stored at −15°C until used.

Sedimentation-viscosity. The purified luciferase was dissolved in 0.1 M NaCl and dialyzed against 4 changes of two liters each of 0.1 M NaCl at 4°C. The solution was then centrifuged at approximately 9000 × g before being used. Sedimentation was run on 5 different concentrations of luciferase, each concentration being prepared by diluting the original solution with the last change of 0.1 M NaCl used in the dialysis. Sedimentation was carried out at 59,780 rpm.[6] between 5–7°C. Five photographic exposures were recorded on Kodak metallographic plate at 32-minute intervals for each concentration of luciferase studied. The plates were subsequently read using a micro-comparator.[7] The luciferase concentration was determined by the methods of constant dry weight and differential refractometry at 436 mμ (Brice and Halwer, '51).[8] Appropriate corrections were made for the presence of NaCl. Viscosity was measured in an Ostwald viscosimeter with an outflow time of approximately 100 secs. for 0.1 M NaCl. The density used to calculate the partial specific volume was determined in a pycnometer of approximately 15 ml capacity. Temperature for viscosity and density determinations were controlled at 25.0 ± 0.005°C in a constant temperature bath.[9]

[6] Analytical Ultracentrifuge, Model E (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.).

[7] Precision Comparator, Type 829A (David W. Mann, Inc., Lincoln, Mass.).

[8] Brice - Phoenix Differential Refractometer (Phoenix Precision Inst. Co., Phila., Pa.).

[9] Isotemp Bath (Fisher Scientific Co., Pittsburgh, Pa.).

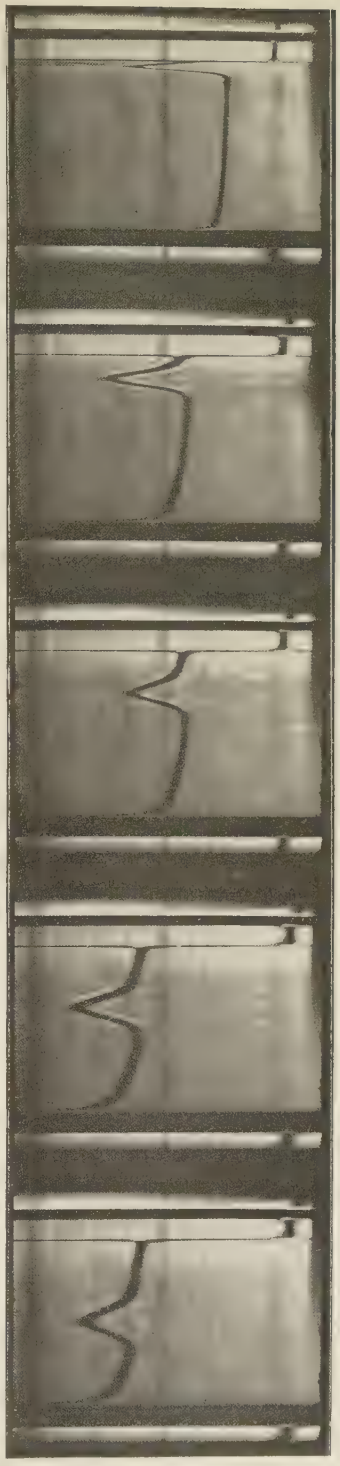

Fig. 1 Sedimentation patterns of purified *Cypridina* luciferase. Concentration of luciferase: 0.141 gm/100 ml of 0.1 M NaCl. Rotor speed and temperature: 59,780 rpm and 5.93°C, respectively. Photographic sequence and direction of sedimentation: right to left. First photograph taken 8 minutes after reaching full speed; subsequent frames at 32-minute intervals. Bar angles employed: 55, 35, 35, 25 and 25°, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the sedimentation pattern of *Cypridina* luciferase at the second highest concentration studied, namely 0.141 gm/100 ml NaCl. The sedimentation patterns at the other concentrations studied, that is, 0.230, 0.089, 0.061, and 0.037 gm/100 ml NaCl, were very similar to the pattern shown in figure 1. Only a single, homogenous boundary was observed during the course of the sedimentation runs.

Figure 2 shows the relationship of the sedimentation constant to luciferase concentration. The sedimentation constants measured in 0.1 M NaCl were corrected to water as solvent at 20°C according to the usual procedure of Svedberg and Pedersen ('40). The sedimentation constants are plotted in Svedberg units (S).

Figure 3 shows a plot of the reciproca of the relative viscosity, $1/\eta_r$, against con centration, C. The equation relating thi term to concentration may be written a follows: (Lauffer, '46.)

$$1/\eta_r = 1 - Ac$$

where A equals the weight intrinsic vis cosity. The relative viscosity was deter mined from its usual relationship to th densities and outflow times of the solven and solution.

The partial specific volume was deter mined according to the method describe by Koenig ('50). The partial specific vol ume was observed to be independent o luciferase concentration. From 5 deter minations performed, a mean of 0.70 cm^3/gm was obtained.

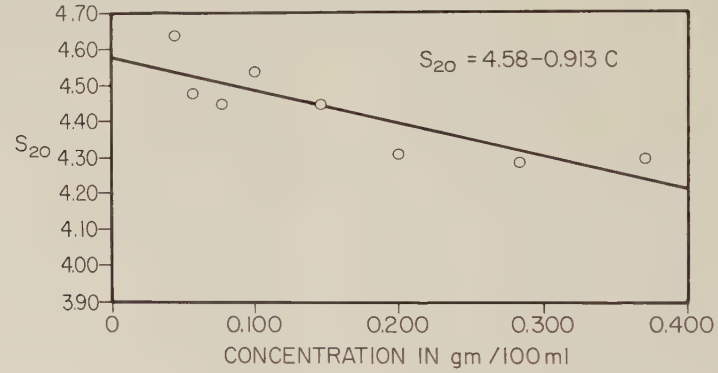

Fig. 2 Variation of sedimentation constant, S_{20}, with concentration, C, for *Cypridina* luciferase, under standard conditions. Line of regress for S_{20} on C determined according to method of least squares. Coefficient of correlation = −0.851; $P = < 0.01$. $S°_{20}$ determined by extrapolating to zero concentration equals 4.58.

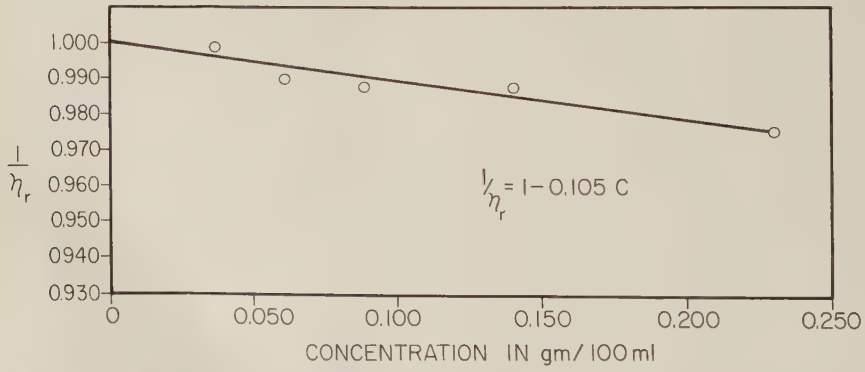

Fig. 3 Variation of reciprocal of relative viscosity, $1/\eta_r$, with concentration, C, for *Cypridina* luciferase in 0.1 M NaCl. Line of regression for $1/\eta_r$ on C determined according to method of least squares. Coefficient of correlation = −0.957; $P \cong 0.01$.

The molecular weight of luciferase may e calculated from sedimentation and vis- sity data using the following equation Lauffer, '44):

$$M^{2/3} = 6\ (f/f_o)\ S^\circ_{20}\pi\eta\ N(3V/4\pi N)^{1/3}/(1 - V_{20}\rho_{20})$$

here

M = molecular weight
f/f_o = frictional ratio calculated from Simha and Perrin equations,
S°_{20} = sedimentation constant at infinite dilution under standard conditions, i.e., in water at 20°C,
η = viscosity of water at 20°C,
N = Avogadro's number,
V = partial specific volume of protein, and
ρ°_{20} = density of water at 20°C.

he frictional ratio may be obtained from ie axial ratio using the Perrin equation Lauffer, '46), assuming an elongated llipsoid of revolution. For luciferase this alue was found to be 1.588. The axial atio, b/a, may be obtained from the vol- me intrinsic viscosity by the Simha equa- on (Lauffer, '46), where b equals the ajor axis and a the minor axis. This atio was found to be 10.89. The volume ntrinsic viscosity in turn may be obtained y dividing the weight intrinsic viscosity y the partial specific volume and multi- lying by 100. This value was found to e 14.85. By making the appropriate sub- itutions, the anhydrous molecular weight as found to be 79,650. The dimensions f the luciferase molecule may also be stimated from the foregoing data since ie volume is equal to $\pi a^2 b/6 = MV/N$ nd the axial ratio equal to 10.89. The orresponding calculated dimensions were 77 A for the major axis and 25.4 A for inor axis. The estimate of the molecular eight is in excellent agreement with the alue of 80,000 obtained by Fedden and hase ('59) from diffusion constant meas- rements using the porous disk method f Northrop and Anson ('29). Chase's 55) earlier estimate of 35,000–39,000 rom sedimentation-viscosity data, how- ver, is not compatible with either of these alues. Assuming a completely spherical olecule lacking water of hydration, Fed- en and Chase ('59) have also estimated eir radius to be 28.8 A.

SUMMARY

A highly purified preparation of lucifer- se has been isolated from the marine crustacean, *Cypridina hilgendorfii*, by fractional precipitation with acetone and ammonium sulfate, followed by gradient elution chromatography on diethylaminoethyl cellulose column. The purified material has been shown to possess a single, homogenous boundary when subjected to sedimentation analysis. Assuming that the luciferase molecule to be represented by an ellipsoid of revolution lacking water of hydration, measurements of the sedimentation constant, viscosity, and density has indicated the molecular weight to be 79,650 and the dimensions of the major and minor axes to be 277 A and 25.4 A, respectively.

ACKNOWLEDGMENTS

We wish to thank Dr. Y. Haneda for supplying us with the *Cypridina* organisms. We are also indebted to the late Professor E. Newton Harvey whose interest in the subject led us to undertake this work.

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Microelectrode Study of Taste Receptors of Rat and Hamster[1]

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The results of electrophysiological research on single fibers of the chorda tympani nerve by Fishman ('57), Pfaffmann ('55), and Cohen et al. ('55) give an insight into the basis of discrimination of the 4 taste qualities. Their reports show that a single fiber may be responsive to more than one or all of 4 basic stimuli. A single fiber of the chorda tympani nerve innervates several taste cells and therefore the response of a single fiber represents the collective properties of several taste cells. It is critical to the analysis of the mechanism of taste stimulation to know whether a single taste cell can respond to taste substances associated with more than one taste quality.

The introduction of microelectrode techniques by Ling and Gerard ('49) has encouraged investigators to study the potentials of sensory receptors. Such potentials have been studied in various sensory cells and the relationship between the potential and the associated nerve discharge has been observed (Granit, '55). Similar microelectrode techniques have been utilized in this study to determine the response of taste cells.

The rat and hamster were chosen as the experimental animals. Taste receptors of both species display high sensitivity to chemical stimulation as compared to those of many carnivores. A response to water, such as that observed with frogs and rabbits, complicates the analysis of taste responses. The rat and hamster show very little or no water response. The hamster was chosen in addition to the rat because of the hamster's good response to sugar stimulation as reported by Beidler, Fishman and Hardiman ('55).

A single taste bud (see fig. 1) is located at the top of almost every fungiform papilla of the tongue of the rat as shown by Fish, Malone and Richter ('44). The taste bud is about 50 μ wide and 60 μ long. Early histologists characterized the cells of the taste bud into two types. The first type of cell, which possesses a round nucleus and a pale cytoplasm, has been called a sustentacular cell. The other type, which is rod shaped with an oval nucleus and dense cytoplasm, was termed a taste cell, since it was similar in appearance to many other sensory cells. Later investigators questioned the advisability of separating the cells into two groups, since many intermediate types could be classified. Parker ('22) felt that new taste cells are continually being formed and that the so-called sustentacular cell is an aged taste cell. Recent electron microscopy by DeLorenzo ('60) and Engstrom et al. ('56) and Murray ('60) indicates that the taste cell is seen in many different forms, possibly due to differences of age. Cells that have degenerated are also seen with the electron microscope. Recent studies with colchicine by Beidler ('60) indicate that the taste cells may continually age and be replaced by new cells.

The double membrane of a taste cell forms gustatory microvilli at the apical end of the cell. Each microvillus is about 250 A in width and 2 μ in length. The so-called taste hairs were not observed with the electron microscope by DeLorenzo ('58).

METHOD

Adult male and female hamsters and albino rats were utilized in these experiments. The animal was anesthetized with

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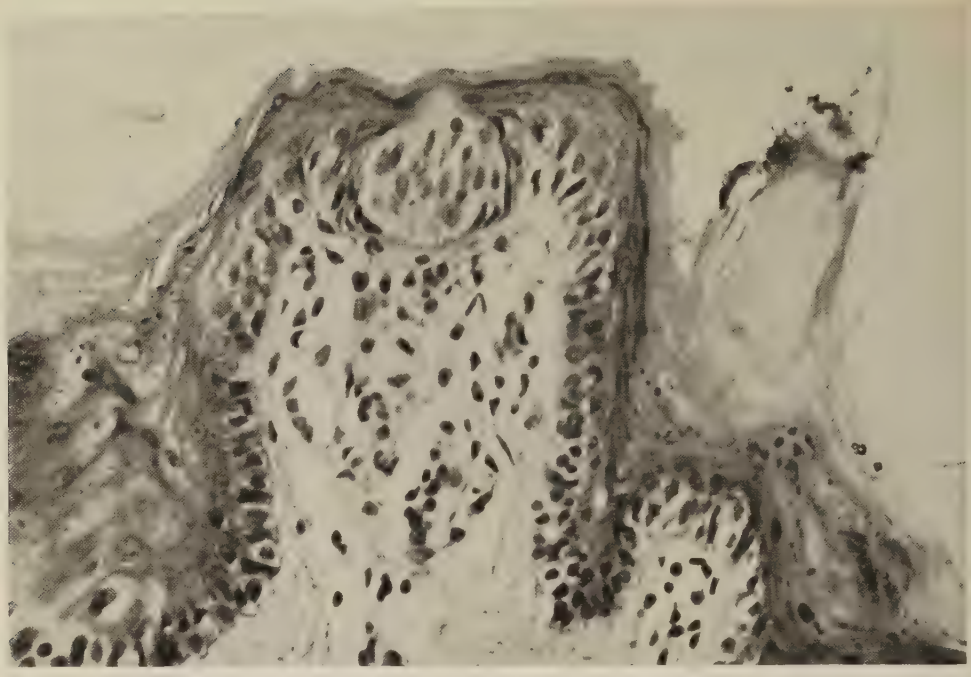

Fig. 1 Fungiform papilla of rat with taste bud close to top surface. Cross-sectional diameter of taste bud is 50 μ.

urethane, placed on an animal board with a head-holder, and the trachea was cannulated. The tongue was pulled out and pinned at the tip. Methylene blue was spread over the surface of the tongue and the white dots indicating the location of fungiform papillae were observed.

The micropipette electrode with a tip diameter of about 0.5 μ was filled with three molar KCl and thrust into the fungiform papilla of the animal under the stereomicroscope. The placement of the electrode was accomplished with the aid of a deFonbrune micromanipulator. A wick electrode on the neck muscle served as the indifferent lead. Both it and the microelectrode with an inserted platinum wire were connected to a DC preamplifier designed by MacNichol ('54). The change in potential was recorded by an ETC oscilloscope and a Sanborn recorder. A voltmeter in parallel with the recorder was used for monitoring. Test solutions were slowly applied to the tongue with the aid of a medicine dropper. Mechanical artifacts were eliminated by applying the solution near the papilla and allowing the solution to flow slowly over the area to b stimulated rather than by applying th stimulus directly on the electrode area As a consequence, no meaningful laten periods could be observed.

RESULTS

Since the individual cell of the tast bud cannot be seen at the surface of th tongue, it is necessary to use functiona criteria as to when the microelectrode pen etrates the taste cell. As the microelec trode penetrates the aqueous layer cover ing the surface of the tongue, a change i potential is observed. The magnitude o this potential depends upon the chemica constitution of this surface layer. Such potentials are purely physicochemical an of little interest to the investigator. It ca easily be seen from figure 1 that if th microelectrode is inserted somewhere a the top center of the fungiform papilla there is only a small probability that th electrode will enter a taste cell. This diff culty is accentuated by the rod-like shap of the taste cell. After the microelectrod is thrust into a fungiform papilla, a nega

tive deflection from 30 to 50 mv may be observed and is considered to be the resting potential of a cell. If this potential does not change when test solutions are applied to the tongue, the above procedure is repeated on another papilla.

The identification of the particular type of cell or receptor that initiates an observed electrical response to a chemical stimulus is always made functionally. For example, a single nerve fiber dissected free from the chorda tympani nerve is assumed to be a taste fiber (and not a temperature, tactile, pain, etc., which also are found in the same nerve bundle) if it responds to low or moderate concentrations of appropriate taste stimuli applied to the surface of the tongue. A similar criterion was established for the purpose of this research. If a microelectrode measures a sudden decrease in DC potential while being thrust through the top of a fungiform papilla, then it is assumed that the electrode has entered a cell. If this DC potential changes with various taste stimuli applied to the surface of the tongue near the entrance of the microelectrode, then it is assumed that this active cell is a taste cell and the depolarization acts as the receptor potential. A large number of attempts were usually made before such a cell was found. Furthermore, no appreciable response has ever been observed when the microelectrode was deliberately thrust into an area devoid of fungiform papillae. The maximum duration of an experiment varied from a few minutes to several hours, depending upon animal movements and the mechanical stability of the recording apparatus.

Rapid initial decrement

Recordings of both single fiber and total nerve activity of taste receptor origin indicate an immediate high magnitude of response followed by a rapid initial decrement lasting about two seconds followed by a rather steady level of activity. The microelectrode recordings from taste cells show neither the immediate high response nor the rapid initial decrement (see figs. 2 and 3). This suggests that the origin of both events is at the site where the action potentials are generated.

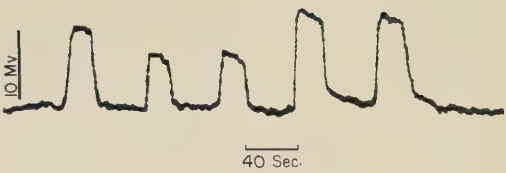

Fig. 2 Receptor potential of a rat taste cell in response to 0.1 M of NaCl, KCl, NH_4Cl, $CaCl_2$, and $MgCl_2$ applied to tongue surface with water rinses between stimuli.

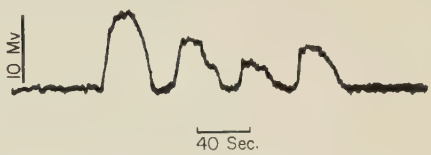

Fig. 3 Receptor potential of a hamster taste receptor in response to 0.1 M NaCl, 0.02 M quinine hydrochloride, 0.5 M sucrose and 0.01 M HCl applied to tongue surface with water rinses between stimuli.

The receptor potential recorded in response to chemical stimulation is very similar in appearance to that of an integrated response of the total taste nerve activity. The similarity is misleading, however, since in the former recording, direct-coupled amplifiers are used throughout and only the Sanborn recorder limits the speed of response. This recorder has a flat response to 35 cps and then drops to 50% at about 80 cps. The integrated responses are usually recorded with a capacitor-coupled amplifier and an integrator with a time constant of about 5 seconds. Thus, the integrated response with a long time constant may reveal no rapid initial decrement and therefore this integrated response may resemble the response recorded with microelectrodes inserted into a single taste cell.

It is noteworthy that no spikes indicative of nerve activity are observed in the taste receptor responses displayed on the oscilloscope. This is probably due to the high noise level associated with the use of micropipette electrodes of high electrical resistance, the high resistance of the cell membrane interposed between the microelectrode and the site of the neural activity, and the large distance (40–50 μ) between the recording location and the site of neural activity.

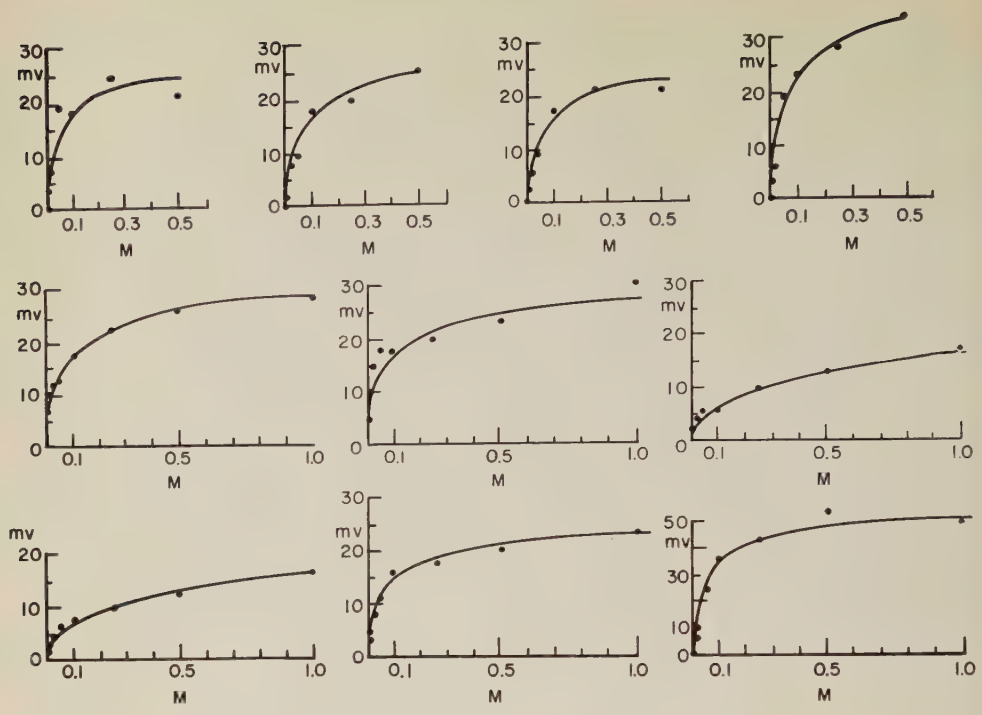

Fig. 4 The receptor potential of each of 10 different taste receptors is plotted as a function of molar concentration of NaCl applied to the tongue. Ordinate: receptor potential in millivolts. Abscissa: NaCl concentration in moles per liter.

Response to sodium chloride

Potential changes are observed in response to various concentrations of sodium chloride applied to the surface of the tongue when the microelectrode is thrust into a cell of the taste bud. In each such case the response to concentrations lower than 5 mM sodium chloride was in the noise level. Larger responses are observed as the concentration is increased. The response usually saturates at a half to one molar sodium chloride concentration. The concentration curves obtained from a number of different taste cells are shown in figure 4. Each taste cell differs in sensitivity to sodium chloride in a manner similar to that observed by Fishman ('57) with single fiber preparations.

At first glance the concentration curve obtained from any single taste cell does not look similar to the concentration curve obtained from the population of receptors by recording the total summated electrical neural activity of the chorda tympani nerve as published by Beidler ('54). However, if the individual responses at each given concentration are algebraically summated, the resulting curve is almost identical to the integrated curve as illustrated in figure 5.

Beidler ('54) considered sodium ions to be bound to a set of equivalent and independent sites located on the receptor surface. Such a consideration led to the derivation of a fundamental taste equation,

$$\frac{C}{R} = \frac{C}{R_s} + \frac{1}{KR_s}$$

where C is the concentration of the stimulus, R is the magnitude of response, R_s is the maximum response at high stimulus values, and K is the equilibrium constant of the adsorption reaction. If this fundamental taste equation is sufficient to describe the magnitude of cell depolarization in response to chemical stimulation, then a plot of C/R vs. C should reveal a straight line. Figure 6 illustrates the goodness of fit

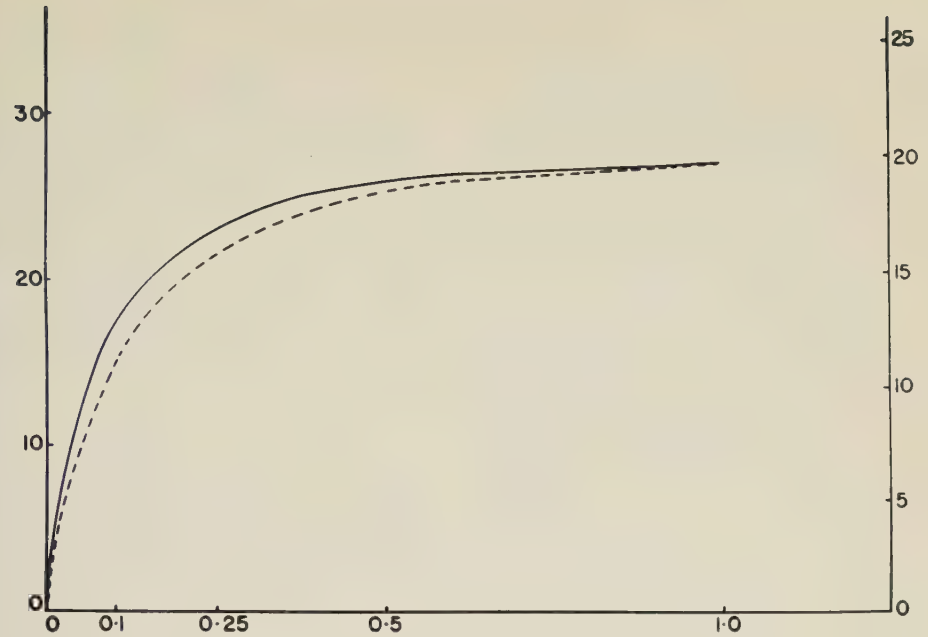

Fig. 5 A comparison of the summated value of receptor potentials (solid line) of 10 taste receptors with the integrated neural activity (dashed line) of the chorda tympani (taken from Beidler, '54) in response to various concentrations of NaCl. The curves have been adjusted to the same asymptote. Left ordinate: receptor potential in millivolts. Right ordinate: relative magnitude of integrated response from taste nerve. Abscissa: molar concentration.

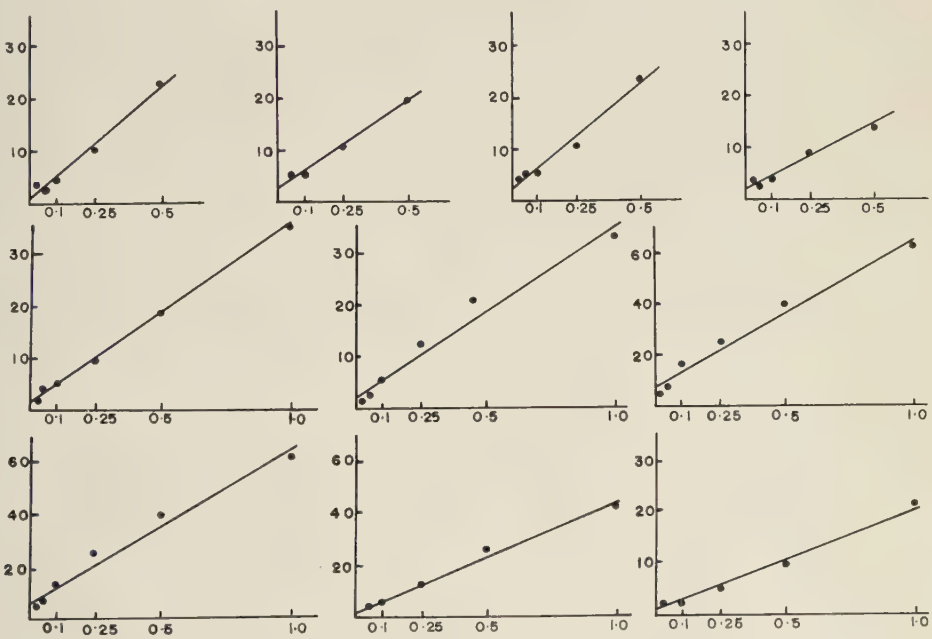

Fig. 6 The receptor potential divided by molar concentration is plotted as a function of molar concentration. Data taken from figure 4.

Response to chloride salts

Responses of a single taste cell to 0.1 M solution of sodium chloride, potassium chloride, ammonium chloride, calcium chloride and magnesium chloride are shown in figure 2. The particular concentration, 0.1 M, was chosen because it produces a response about half of the magnitude of the response to very high concentrations. The response to sodium chloride is relatively larger than that to the other salts. Sometimes calcium chloride and magnesium chloride produce larger potential changes than sodium chloride. Similar results have been observed by Fishman ('57) in recording from single fibers. Table 1 shows that individual taste cells are different in responsiveness to different kinds of salts. However, the summation of these results should approach the relative magnitudes as obtained from the integrated electrical activity of the chorda tympani nerve. This is not always the case, however.

Response to sodium compounds

Beidler ('53) has shown that the responsiveness of taste receptors of the rat is primarily due to the excitatory effect of the cation, although the anion has a small inhibitory effect which increases with the chain length in a homologous series. To compare receptor potential responses with those from the nerve, solutions of 0.1 M concentration of sodium chloride, acetate, citrate, formate and propionate were used as test solutions. The magnitudes of responses of the taste receptor to these sodium salts were very similar, although the responses to sodium propionate and acetate were consistently smaller than to sodium chloride and sodium formate. Thus the integrated data of Beidler ('54) and the taste receptor data agree well and indicate that although the relative response of a taste receptor to a number of different cations varies from one receptor to another, the order of sensitivity to an homologous series of anions is similar for each cell studied.

Prolonged discharge

Beidler ('53) has shown that after two seconds the nerve activity remains at a constant magnitude as long as sodium chloride is flowed over the surface of the tongue. However, the response to calcium chloride steadily declines. The single taste cell depolarization is maintained with sodium chloride and the response to calcium chloride reveals only a slow decline and is not similar in appearance to that often obtained from the taste nerve activity. This suggests that some chemicals may react eventually with the receptor or nerve fiber at a place other than at the receptor surface such that the nerve activity reflects events in addition to the normal receptor depolarization. Such discrepancies are rarely seen, however, particularly when high stimulus values are avoided.

TABLE 1

Response in millivolts of rat taste receptor to 0.1 molar chloride salts

Taste receptor	NaCl	KCl	NH_4Cl	$CaCl_2$	$MgCl_2$
1	13	8.5	9.5	13	15.5
2	9	6	7.5	17.5	16
3	5.5	2	7.5	5.5	5
4	6	2	6	5	5.5
5	5.5	5	6.5	4	2.5
6	5	3	4	7.5	5
7	5	2	3	4	6
8	6	3	4	6	6
9	6	5	5.5	7	4
10	7	4.5	4	7	6.5
11	8.5	10	15.5	6	10
12	12.5	9.5	15	8	5
13	11	9.5	8.5	10.5	6.5
14	10	6	6.5	12.5	8.5

TABLE 2

esponse in millivolts of rat taste receptor to basic stimuli of various taste qualities

Taste receptor	0.1 M NaCl	0.5 M sucrose	0.02 M quinine hydrochloride
1	23	0	14
2	10	0	6
3	18	10	20
4	15	4.5	5.5
5	22	2	16
6	13	5.5	9
7	6	2.5	7
8	6	1.5	3
9	7	1	4
10	7	1	3
11	8	1	8.5
12	8.5	2.5	3
13	8	0	7
14	10	12	5
15	12.5	1.5	2.5
16	11	2.5	7.5
17	10	5.5	11.5

Response to 4 taste qualities

One-tenth molar sodium chloride, 0.02 I quinine hydrochloride, 0.5 M sucrose nd 0.01 M hydrochloric acid were used s basic stimuli for the 4 taste qualities—alty, bitter, sweet and sour. Potential hanges recorded with the microelectrode nserted in the taste receptor are shown n figure 3. Each receptor is responsive o stimuli of more than one taste quality; urthermore the relative magnitudes of re-ponse vary from one receptor to the next see tables 2 and 3). These results all ppear to be similar to the data obtained rom single taste fiber studies.

DISCUSSION

The concept of 4 taste qualities has lways been of concern to physiologists nterested in taste. It has always been hoped that some specific anatomical structures would be specific to one taste quality and not to others. Thus it was hoped a simple physiological mechanism could be formulated explaining the discrimination of taste qualities. Such has not been the case, however. Kiesow (1894) and Oehrwall (1891) demonstrated that the cluster of taste buds on a single human fungiform papilla was responsive to more than one taste quality. Many years later new electrophysiological techniques enabled Pfaffmann ('41), Cohen et al. ('55) and Fishman ('57) to investigate the response of single taste fibers. Here also it was found that most single taste fibers responded to stimuli representative of more than one taste quality. Since some of these experiments were performed in the rat where single taste buds are separated by rather large distances, it could be assumed that a single taste fiber innervated cells of only one taste bud. However, the fiber innervates more than one taste cell within that bud. Present microelectrode investigations have revealed that a receptor also responds to stimuli associated with more than one taste quality. Thus the problem of taste discrimination is still unsolved. Furthermore, the analysis of the mechanism of taste stimulation must take into account the fact that more than one type of taste receptor site exists on a given receptor cell. How the higher nervous centers discriminate information concerning taste qualities that arise from the complex taste receptors is not yet clear.

Data obtained from microelectrode studies are very similar to those derived from the measurements of the integrated response of the chorda tympani nerve or single fiber data by Fishman ('57) and

TABLE 3

Response in millivolts of hamster taste receptor to basic stimuli of 4 taste qualities

Taste receptor	0.1 M NaCl	1.0 M sucrose	0.02 M quinine	0.01 M HCl
1	10.5	2	4.5	—
2	8.5	2	7.5	0
3	9	1	4.5	—
4	11.5	5	8	22.5
5	10	6.5	9	12.5
6	6.5	1.5	5	—
7	9.5	2.5	7	9
8	14	0	5	14

Pfaffmann ('55) with respect to the response to various sodium chloride concentrations and the response to a chloride series of sodium salts.

Data obtained with both techniques are adequately described by the taste equation previously derived by Beidler. The values of the equilibrium constant, K, obtained from single cell responses to NaCl were found to range from 7.1 to 40.5 with a mean of 16.7 in contrast to the mean value of 9.8 previously found by Beidler ('54) using integrated responses of the whole chorda tympani. The difference in the response curves can be seen in figure 5. It should be noted that taste receptors with low values of K would be expected to respond with small changes in resting potential. Since the limit of response detection is set by the drift or instability of the electrodes and DC amplifier, such taste receptors would probably not be studied with the techniques used in this study. Thus, the K values in the present study are biased toward high magnitudes.

Agreement between the data obtained from microelectrode studies and from integrated responses to different taste qualities is not too good, although several explanations may be offered. First, most microelectrode studies were performed for convenience on the same area of the tongue, mainly a small area at the top surface of the tip. It has been known for a long time that various areas of the tongue respond differently to different taste qualities. The integrated technique reveals the total response of receptors on the front two-thirds of the tongue. The single fiber technique selects receptors of the tongue at random. Second, the comparison is made with too small a number of observations in both the case of microelectrodes and single fibers. Furthermore, when the recordings are made from single taste fibers there may be a contribution from nerve ramifications that do not enter a taste bud. It is also possible that some cells of the taste bud respond to solutions by partially depolarizing, even though the cells may no longer be innervated by many taste nerve fibers. This would seem particularly true if such cells are really transitional taste cells as suggested by Parker ('22) and Beidler et al. ('60).

Since the same general equation,

$$\frac{C}{R} = \frac{C}{R_s} + \frac{1}{KR_s},$$

adequately describes the quantitative da obtained from observing the changes i resting potential as well as the data o tained from observing the magnitude neural activity, it appears most likely th the frequency of steady state nerve di charge is related linearly to the magnitu of the receptor potential. However, sin the stimuli to these cells are chemical i nature, it should be expected that certai chemicals may interfere with various pro esses within the taste cell or synapt junction. Such may be the case of calciu chloride where the taste cell potential do not decrease rapidly with prolonged stim ulation as does the neural activity r corded from taste nerve. Anatomists ha shown that a single taste fiber near t taste bud divides into a number of te minal branches, most of which enter t taste bud and innervate the taste cell but some of which end as free nerve en ings in the surrounding epithelial ner tissue. The contribution of such free en ings to single taste fiber data is not know

What is a reasonable explanation f the mechanism of taste receptor stimul tion and resultant neural discharge? T taste receptor cell is a specialized epith lium cell located in the taste bud. The i dividual cells of the taste bud degener rapidly and are continually replaced. Ea taste cell has numerous microvilli whi project into the saliva covering the tongu These microvilli are probably the chem cally sensitive surfaces of the taste cell. T receptor surface contains many differe kinds of sites to which ions and molecul can be adsorbed by weak forces (Beidle '54). Some sites are excitatory in natu and others inhibitory. The distribution different excitatory sites varies from on taste cell to another and the number sites of any one type averaged over all t taste cells also varies from one species animal to another (Beidler et al., '55 The taste receptor depolarizes as cation or molecules are adsorbed to the excitato surface, presumably the microvilli. Th depolarization initiates, either by electric

chemical measures, a graded response the unmyelinated terminal branches of e taste nerve. Each taste nerve fiber vides into many (4–15) such branches it leaves the nerve plexus under the ste bud and enters the bud between the ste cells. The various graded electrical sponses may be conducted with a decre- ent down the various branches, combine eir influence when meeting at a junc- on, and initiate an all or none nerve im- lse at the first node of Ranvier. The ove explanation may be used for further perimentation and possible verifications.

SUMMARY

Slow potential changes of the taste re- ptors elicited by a number of salts or imuli representing the 4 taste qualities ere observed by using a micropipette ectrode thrust into the taste bud located a fungiform papilla of the front por- on of the tongue of the rat and hamster. he results indicate that the receptor is sponsive to one or more of the stimuli presenting the 4 taste qualities and is ot specialized to be sensitive exclusively but one type of chemical stimulus. here is also variability in sensitivity of ie taste receptors to the stimuli of 4 taste ualities and to the chloride salts, al- ough the response to sucrose was diffi- ult to observe. These results are similar the data of the single fibers studies of ie chorda tympani nerve.

Results obtained are not always similar the data of the integrated response of ie chorda tympani nerve and the reason or this disagreement has been discussed.

ACKNOWLEDGMENTS

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Membrane Activity of Chronically Denervated Frog Sartorius Muscle Fibers [1]

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Recent studies have revealed that the major factor contributing to the observed chemical supersensitivity of a chronically denervated skeletal muscle fiber is a progressive increase in the size of the membrane area which is responsive to locally applied acetylcholine (ACh), from one which is restricted to the immediate vicinity of the endplate to one which eventually involves the entire fiber (Ginetzinsky and Hamarina, '42; Axelsson and Thesleff, '59; Miledi, '60). Further the ACh receptors which appear after denervation possess properties which are similar to those of the endplate receptors. These observations raise the question of whether such an acquisition of chemosensitivity by the non-endplate membrane results in an alteration of the normal electrical excitability of this membrane.

It has been concluded that the endplate membrane of the frog twitch fiber is electrically inexcitable, i.e., that it does not possess the mechanism for the production of an action potential (Grundfest, '57; Werman, '60). However Nicholls ('56) has demonstrated that the non-endplate membrane of denervated frog skeletal muscle remains electrically excitable. It appears therefore that the structures responding to chemical stimuli and those which are capable of electrical excitation, may exist intermingled in the skeletal muscle membrane.

Available evidence suggests that the non-endplate membrane of denervated skeletal muscle becomes chemosensitive as a result of an increase in the number of ACh receptors there (Miledi, '60, '61). This raises the possibility that ACh receptors proliferate at the expense of the electrically excitable structures, either as a result of transformation or displacement. Since an appreciable reduction in the density of electrically excitable elements would be expected to lead to certain quantitative changes in the action potential, a study has been made of several critical features of the action potential in the denervated frog sartorius muscle. Data presented indicate that despite its increase in chemosensitivity, the non-endplate membrane of the denervated skeletal muscle fiber retains its ability to produce an action potential indistinguishable from that recorded in an innervated fiber. Some of these findings have been reported in preliminary form (Levine, '59).

METHODS

Biological material. All of the experiments reported were performed on the isolated sartorius muscle of *Rana pipiens*, and were conducted at room temperature (21–26°C). The denervation procedure consisted of anesthetizing each frog with 1–2 ml of a 10% urethane solution injected into the ventral lymph sac, separating the coccygeo-iliacus muscle bluntly along one side of the urostyle, and removing a length of spinal nerves 7–9 of approximately 1 cm. The wound was lightly dusted with sulphanthiazole powder and the skin closed with a single silk suture. Mortality did not differ from that of unoperated frogs kept for the same length of time. All frogs were maintained without feeding in a bin with a water temperature of 18–23°C for a minimum of three weeks prior to study. For most studies both sartorii of pithed

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frogs were removed by careful dissection, the denervated muscle being examined directly and the normal muscle being stored at ca. 5°C in Ringer's solution for 4–24 hours prior to study.

Solutions and microelectrodes. In all important respects the microelectrode techniques, composition and changing of the Ringer's solution bath, and the mounting and visualization of the sartorius muscle were identical to those described by Nastuk ('53). Only microelectrodes of 6–15 megohms resistance and with tip potentials of less than 5 mv were used (Adrian, '56). These characteristics were checked at frequent intervals during the course of each experiment, and microelectrodes were replaced if either resistance or tip potential changed appreciably.

Recording equipment. The input unit was the slightly modified "negative capacity" preamplifier designed by Solms, Nastuk and Alexander ('53). Prior to impalement of any cell whose action potential (AP) was to be recorded, the system was critically compensated by the procedure outlined in the above reference, so that all AP records were made on a system having an overall time constant of less than 10 μsec. Input cathode follower grid current was 10^{-12}a or less. This preamplifier fed a differential DC amplifier of conventional design, the output of which was coupled to a dual beam oscilloscope having independent time bases. The output voltage of the DC amplifier also controlled the tone frequency of an audiomonitor, so that membrane impalements could be performed under direct visualization and an audio cue signalled the instant of contact with the intracellular fluid. Only those insertions were accepted in which penetration of the membrane was made without apparent dimpling and in which the change in tone of the audio monitor was abrupt and maintained.

Records. The voltage calibration was achieved by recording a raster of 10 or 20 mv steps applied to the bath electrode prior to each implement, so that every record consisted of a resting potential (RP) and an AP superimposed on a voltage calibration grid. The zero potential base line was always recorded a second time immediately after withdrawing the microelectrode from the cell. Timing marks (usually 0.1 msec.) were applied to the base line of each re cord when the voltage grid was recorded Photographic records of oscilloscope trace were made on 35 mm film and were ana lyzed by projection onto graph paper.

Accuracy of AP measurements. Com putation by the method described by Solms Nastuk and Alexander ('53) shows that system having an input time constant c 10 μsec. and no appreciable distortion in troduced by succeeding stages, will recor the amplitude of an AP in a sartorius mus cle fiber without measurable error, and wi follow the maximum rate of rise of the A with an error which is within the limits o resolution of the method of graphical ana ysis employed. Slope measurements mad on records of calibrated sine waves applie to the input circuit under recording condi tions indicated an accuracy of ± 3% fo the method used.

Acetylcholine sensitivity. The degree o supersensitivity of the denervated muscle was evaluated by determining the ACh thresholds for single fibers in normal an denervated muscles of the same animals ACh threshold was taken as the minima concentration of ACh required to evoke contractile response. Details of the proce dure have been described elsewher (Nastuk and Levine, '61).

Statistical analyses. In the experiment where a comparison was made betwee properties of the two sartorii of the sam frog, or where endplate and non-endplat properties of the same muscle were com pared, the accumulated data were proc essed statistically by calculating the mea difference of the pairs in the series. I other cases the difference between th overall means of the series was tested fo significance. Student's t-test was employe throughout.

Terminology. Figure 1 is a tracing of typical RP and AP record with the variou parameters measured in this study indi cated. Abbreviations used in this paper ar listed.

RESULTS

Membrane potentials from the two sartorius muscles of the same frog

An individual fiber was impaled at non-endplate site and the RP recorded

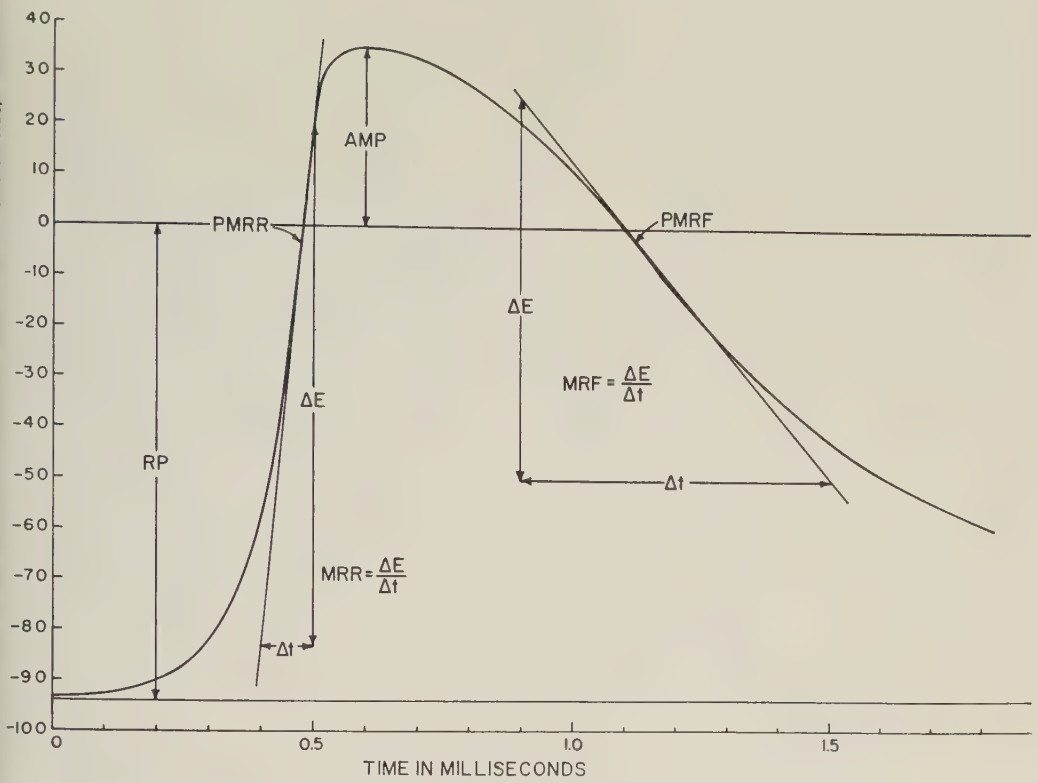

Fig. 1 Tracing of a typical action potential from a frog sartorius muscle fiber recorded at a non-endplate site in response to direct electrical stimulation. The various parameters studied are indicated by the symbols: RP, resting potential; MRR, maximum rate of rise; PMRR, potential difference across membrane at MRR; AMP, active membrane potential; MRF, maximum rate of fall; PMRF, potential difference across membrane at MRF. Zero on time axis arbitrarily chosen.

The fiber was then directly stimulated at a site 1 mm distant from the intracellular electrode and the propagated AP was recorded simultaneously on the two beams of the oscilloscope. One trace had a duration of 5 msec. and recorded the entire AP with the exception of the slow after potential; the other trace had a duration of 0.8 msec. and was appropriately delayed to record only the rising phase of the AP, in order to facilitate subsequent graphical analysis. Stimulation was achieved by means of a pair of external electrodes and was limited to only a few fibers at a time by using for one of the electrodes a stainless steel wire ground to a tip of ca. 10 μ and insulated throughout except for a small pore at the extreme tip. The critical membrane potential (CMP) was recorded by placing the steel electrode to within 50 μ of the recording microelectrode and applying successively greater rectangular pulses of 0.1 msec. duration. The point of inflection between the local response and the propagated AP was taken as the CMP, with the requirement that at least 0.1 msec. must elapse between the end of the stimulus and the initiation of the AP. Recording of just subthreshold stimuli with the microelectrode immediately outside the fiber, indicated that after this time interval had elapsed, less than 1 mv of potential difference existed in the extracellular fluid between the point of recording and the bath electrode.

Unoperated frogs. Since both sartorii were to be dissected at the same time from each operated frog, but had to be studied sequentially, sometimes with an interval of 24 hours, a preliminary control study was made on 7 pairs of sartorius muscles from unoperated frogs. Also since the

studies on the denervated muscles were to be made on animals which had been received into the laboratory at least three weeks earlier, this control study was performed on unoperated frogs which had been on hand for ca. 1 month. In the experiments on the unoperated frogs the muscle from the left side was always studied first.

Data obtained from the two normal muscles of unoperated frogs reveal no mean difference between the right and left sartorii with respect to any of the characteristics examined. These data have been summarized in table 1 by presenting for each parameter its overall mean and standard error. Thus a dissected normal frog sartorius muscle may be stored at ca. 5°C for at least 24 hours without alteration of a number of physiologically important membrane characteristics.

Operated frogs. In this series the denervated muscle of each pair was always examined first. Table 2 summarizes the data obtained from 6 frogs in which the left sartorius muscle had been denervated 3–8 weeks previously. Statistical analysis of these data likewise reveals no mean difference between the denervated and the innervated sartorius muscles of the same frog for any of the characteristics studied. Thus for at least two months following denervation, the skeletal muscle membrane preserves the mechanisms for the maintenance of a normal RP and the generation of a normal AP. The finding that neither the RP nor the CMP of denervated muscle differs from the corresponding value recorded in innervated muscle is of some interest, since it eliminates the possibility that a reduction in the critical depolarization can contribute to the mechanism of supersensitivity.

Comparison of the membrane potentials of muscles from operated and unoperated frogs. The previous statistical study, in which the innervated muscle serves as a control for the denervated muscle of the same animal, does not eliminate the possibility that membrane potentials of *both* muscles in operated frogs may differ from

TABLE 1

Comparison of membrane potential characteristics of innervated sartorius muscles of unoperated frogs

Characteristic	Left sartorius[1]	Right sartorius	P[2]
RP (mv)	−88.0±0.9 (160)	−88.6±1.1 (142)	> 0.7
AMP (mv)	+35.6±1.3 (109)	+35.3±1.2 (119)	> 0.6
MRR (v/sec.)	754±31 (78)	759±30 (84)	> 0.8
PMRR (mv)	−10.1±1.6 (78)	−11.7±1.3 (84)	> 0.2
MRF (v/sec.)	129.6±5.2 (80)	123.5±6.1 (86)	> 0.4
PMRF (mv)	−1.0±0.9 (80)	−0.8±0.8 (86)	> 0.8
CMP (mv)	−44.1±1.0 (61)	−43.8±1.1 (67)	> 0.5

[1] Each datum is the mean of 7 muscles ± S.E. Figures in parentheses give number of individual fibers. Membrane potentials expressed as inside potential minus outside potential.
[2] Probability values calculated from the mean differences of individual pairs.

TABLE 2

Comparison of membrane potential characteristics of chronically denervated and contralateral innervated sartorius muscles

Characteristic	Denervated sartorius[1]	Innervated sartorius	P[2]
RP (mv)	−86.9±1.1 (95)	−88.7±1.2 (97)	> 0.2
AMP (mv)	+37.2±1.5 (62)	+37.4±1.1 (72)	> 0.7
MRR (v/sec.)	525±20 (55)	540±18 (70)	> 0.7
PMRR (mv)	−9.4±1.4 (55)	−11.6±1.2 (70)	> 0.1
MRF (v/sec.)	73.3±2.8 (61)	80.7±2.6 (68)	> 0.1
PMRF (mv)	−1.0±1.0 (61)	−0.9±0.7 (68)	> 0.8
CMP (mv)	−46.4±1.6 (42)	−44.2±0.8 (53)	> 0.2

[1] Each datum is the mean of 6 muscles ± S.E. Figures in parentheses give number of individual fibers. Membrane potentials expressed as inside potential minus outside potential.
[2] Probability values calculated from mean differences of individual pairs.
Duration of denervation: 3–8 weeks.

ose of muscles of unoperated frogs. A ries of measurements was made on the P's of fibers of three normal sartorius uscles of freshly received frogs, and the ean value was -92.7 ± 0.5 mv. This lue is in line with recently reported de- rminations of the RP of frog sartorius uscle where precautions have been taken minimize microelectrode tip potentials Nastuk, '53; Adrian, '56; Hodgkin and orowicz, '59). However it is significantly rger ($P < 0.01$) than the mean RP found the denervated muscle as well as the ean RP found in the contralateral in- rvated muscles of operated frogs. This wer RP found in the muscles of operated ogs may be related to the generally poor utritional condition of these animals, hich had been maintained in the labora- ry for long periods and which were nfed.

A comparison between the mean values ported in table 1 and the corresponding eans given in table 2 reveals two striking fferences. In both muscles of the oper- ed animals the MRR and the MRF of the P are lower by 30% and 40% respec- vely, than the MRR and the MRF of the uscles of the unoperated frogs. Both of ese differences are statistically signifi- nt ($P < 0.01$).

From a knowledge of the MRR and the RF and the membrane capacity, the ionic rrent across the membrane may be cal- lated at the points of inflection of the P (Hodgkin and Katz, '49). Since the embrane capacity is unchanged in de- rvated frog muscle (Nicholls, '56), an verage value of 8 μfd/cm² may be taken r both normal and denervated muscle Katz, '48; Fatt and Katz, '51; Tasaki and agiwara, '57). Currents so calculated for e muscle from unoperated frogs are 6.0 a/cm² for the rising phase, and 1.0 ma/ m² for the falling phase of the AP. Cor- sponding values for the muscle from erated frogs are 4.3 ma/cm² and 0.6 a/cm².

It is reasonable to interpret the observed duction in inward current during the ris- g phase of the AP as a diminution of the dium ion current (Nastuk and Hodgkin, 0). This would mean that during the sing phase of the AP there is a reduction the driving force acting on the sodium ions or a decreased elevation of the permeability of the membrane to sodium ions, or both. The permeability of the membrane to sodium ions may be defined in terms of the sodium conductance, g_{Na}, as

$$g_{Na} = \frac{I_{Na}}{E - E_{Na}},$$

where I_{Na} is the sodium current, E is the potential difference across the membrane, and E_{Na} is the sodium ion equilibrium potential (Hodgkin and Huxley, '52). Since the membrane potential at which the MRR occurs is not changed (PMRR, tables 1 and 2), any reduction in the driving force acting on the sodium ions would have to result from a decrease in E_{Na}. Using the values reported in this paper for E and I_{Na}, and a value of + 52 mv for E_{Na} for muscle from unoperated frogs (Nastuk and Hodgkin, '50), the value of E_{Na} for the muscle from operated frogs is calculated as + 34 mv, *if* it is assumed that g_{Na} is the same in muscles from operated and unoperated frogs. Since the average value for AMP in muscles from operated frogs has been found to be + 37 mv (table 2), the reduction of I_{Na} in these muscles cannot result simply from a decrease in E_{Na}. It may be concluded therefore that the membrane permeability to sodium ions during the rising phase of the AP is significantly reduced in the muscles from the operated frogs.

A similar analysis for the observed reduction in MRF is not possible because the available data do not permit a choice between the alternative possibilities of a decreased potassium ion current and a delay in the inactivation of the sodium ion current.

Sensitivity of individual fibers to acetylcholine

The development of supersensitivity to ACh has been followed over a period of 7 weeks in a different series of frogs. The minimal concentration of ACh required to evoke a contractile response was determined at intervals following denervation in 6 pairs of sartorius muscles. From 7–13 fibers were tested individually in each muscle. In every fiber tested the ACh threshold was lowest at the region immediately around the endplate. Responses could be elicited at sites remote from the endplate in denervated fibers, but only with 100 to

1000-fold higher concentrations of ACh. Considerable variation in ACh threshold from endplate to endplate existed in each muscle, especially the denervated ones, but there was no overlap in the range of ACh threshold between denervated and normal fibers (fig. 2). Sensitivity continued to increase up to the end of the period of study, and presumably would have shown further increase with time. The data reveal an increase in the absolute sensitivity of the innervated fibers from the control muscles over the course of this time, and after 6 weeks the mean ACh threshold of the innervated fibers differed significantly ($P < 0.01$) from the mean ACh threshold observed on the first day of the study. Therefore the degree of supersensitivity of the fibers denervated for 7 weeks relative to contralateral innervated fibers of the same frog (122 ×), is not as great as that relative to innervated fibers examined at the beginning of the study (333 ×). These results are summarized in table 3 under the heading, "Normal Ringer."

Following the determinations on the ACh thresholds in the normal Ringer's solution, the muscles were soaked for

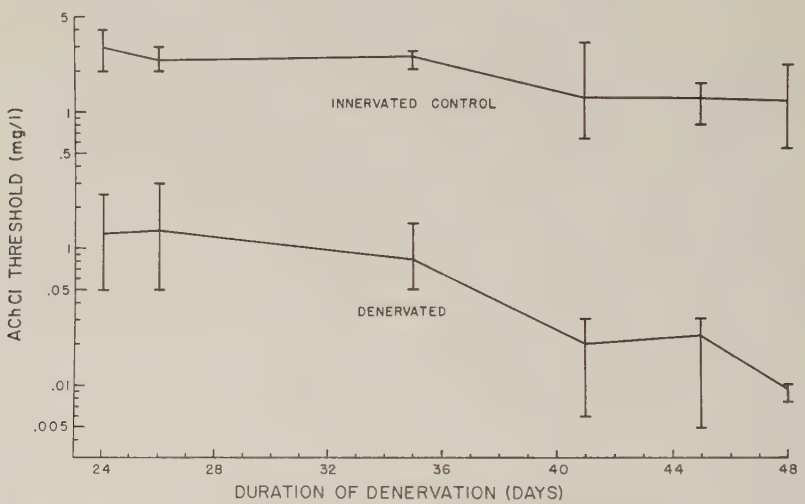

Fig. 2 Sensitivity of denervated and contralateral innervated sartorius muscle fibers to acetylcholine. ACh threshold is taken as the smallest concentration required to cause a visible contractile response. Range of individual fiber thresholds in each muscle is indicated by vertical bar, and the mean values have been joined. Data are from muscles in normal Ringer's solution.

TABLE 3

Degree of supersensitivity[1] to acetylcholine of chronically denervated sartorius muscle fibers

Days denervated	Normal Ringer's			Ringer's + Eserine[2]			Number of fibers	
	Least	Mean	Most	Least	Mean	Most	Denervated	Innervated
24	16	23	40	10	11	50	12	11
26	10	18	40	6	7	12	9	7
35	18	30	40				10	10
41	100	60	100	20	21	30	13	8
45	50	51	150				9	7
48	200	122	67	133	118	43	9	11

[1] Explanation of headings:

Least: ratio of ACh threshold of least sensitive innervated fiber to ACh threshold of least sensitive denervated fiber.

Mean: ratio of average ACh threshold of innervated fibers to average ACh threshold of denervated fibers.

Most: ratio of ACh threshold of most sensitive innervated fiber to ACh theshold of most sensitive denervated fiber.

[2] Eserine concentration: 5 mg/l.

eriod of 30 minutes in Ringer's solution ontaining 5 mg/l of eserine sulphate, in rder to inhibit cholinesterase (ChE) ac- vity. This concentration of eserine was hosen because there is evidence that at omewhat higher concentrations (ca. 30 ng/l), this drug inhibits ACh receptor ac- vity (Eccles, Katz and Kuffler, '42). A econd series of determinations of ACh nreshold was then made on the same fi- ers as had been examined in the normal inger's solution. Results are presented in able 3 under the heading, "Ringer + serine." All 43 fibers examined in 4 in- ervated muscles showed an increased sen- itivity to ACh, the average gain amount- ng to a factor of 3. In contrast, two of the denervated muscles studied showed no nean increase in sensitivity to ACh, and n the other two denervated muscles, the verage gain was less than that seen in ny of the innervated muscles. These find- ngs were expected in view of the reported eduction of ChE activity at endplate re- ions of denervated amphibian muscle Feng and Ting, '38). Similar effects have een reported by Birks, Katz and Miledi '60), who found that neostigmine has a ess marked potentiating effect on the re- umed spontaneous miniature potentials ecorded at denervated endplates of the rog sartorius muscle, than it has on the ormal miniature endplate potentials of nnervated fibers.

These results show that, other factors emaining equal, reduction of ChE activity t the endplate membrane of denervated bers can account for a several fold gain n fiber sensitivity to ACh. This is how- ver only a small fraction of the total in- rease in sensitivity to ACh observed in enervated fibers.

Resting potential of the denervated endplate membrane

It has been reported by Buchthal and indhard ('34) that a potential difference xists between the endplate and the adja- ent membrane in single fibers of inner- ated lizard muscle. Such a potential radient around the endplate membrane as not been observed in normal fibers of he frog sartorius muscle (Kuffler, '43; att and Katz, '51; Nastuk, '53). It seemed f interest to inquire whether a potential difference between endplate and adjacent muscle membrane developed in denervated fibers, since the appearance of such a potential gradient could make a contribution to the mechanism of supersensitivity by reducing the critical depolarization.

A comparison has been made between the RP of the denervated endplate membrane and that of non-endplate membrane of the same fiber. The mean RP recorded at endplate sites has also been compared with the mean RP recorded from a group of fibers at the pelvic end of the same muscle. A difficulty in this determination is the uncertainty involved in identifying the precise location of the denervated endplate membrane. The studies on ACh sensitivity indicated that the surviving neurilemmal sheath could be followed to within 50–100 μ of the endplate, but it was frequently difficult to identify the specific fiber within a circular area of ca. 100 μ diameter on which the endplate was situated. The fiber deemed to be the site of the endplate was impaled and the RP determined. Then the two fibers immediately adjacent were impaled and their RP's recorded. The RP of each of the three fibers was then recorded a second time, at a site approximately 1 mm distant from that of the first impalement. The microelectrode was then moved to the extreme pelvic end of the muscle, and RP's were measured in 15–18 randomly chosen fibers.

One hundred and one impalements were made at 34 endplate sites on 7 denervated muscles (duration of denervation, 3–12 weeks). Of this total only 6 RP's were encountered which differed from the mean RP of the muscle in which they were measured (calculated from the measurements made at the pelvic end) by more than ± 2 S.D. of the mean.

The mean difference between the two RP determinations for the 95 fibers included in the range, mean ± 2 S.D., was − 1.3 ± 0.9 mv (endplate RP − non-endplate RP). The 6 fibers which had endplate RP's more than ± 2 S. D. from the mean RP of their muscle, averaged − 2.2 ± 3.5 mv difference from the RP measured at a site 1 mm distant on the same fiber.

The mean non-endplate RP for 110 fibers in 7 denervated muscles recorded at the pelvic end was − 86.4 ± 1.3 mv. The

mean RP for the 34 endplate sites in the same 7 muscles was -86.3 ± 2.4 mv.

Thus individual RP measurements at endplate locations show only the anticipated statistical scatter about the mean non-endplate RP, and the mean values at the two membrane sites are practically identical. There is no suggestion that a potential difference between endplate and non-endplate membrane develops in denervated frog sartorius muscle.

DISCUSSION

The results of this study clearly show that the RP and AP of the frog sartorius muscle fiber, denervated for as long as two months, do not differ from those of contralateral innervated fibers. Since many of the properties of skeletal muscle are altered at least quantitatively after denervation (Tower, '39), it is of some interest that the mechanisms underlying the maintenance of the RP and the generation of the AP are preserved either unaltered or at a level which does not prevent their normal expression.

The data presented demonstrate that AP's, elicited by direct electrical stimulation of membranes which are also capable of responding to chemical stimuli (Miledi, '60), show no quantitative differences from AP's propagating through membranes which are chemically insensitive. This supports the earlier conclusion of Fatt and Katz ('51) and del Castillo and Katz ('54) that the ACh receptors are electrically inexcitable.

The appearance of ACh receptors in the non-endplate membrane, without demonstrable effect on the operation of the mechanism of electrical excitability, indicates the composite nature of the membrane of denervated muscle. Such a membrane type, with intermingled electrically and chemically excitable components, has been previously described for the innervated eel electroplaque (see Grundfest, '57 for refs.). These observations are consistent with the view expressed by Fatt ('59), that the area occupied by the endplate receptors is only a small part of the junctional membrane.

Data presented in this paper confirm the earlier report of Nicholls ('56) that the RP of frog sartorius muscle is unchanged by chronic denervation. These data however differ from those of Bogatzki ('52) who has reported a progressive decline in the demarcation potential of frog sartorius muscle with time after denervation. Since it is generally agreed that the connective tissue space of skeletal muscle undergoes at least a relative increase following denervation (Tower, '39; Sunderland and Ray, '50; Eichelberger et al., '56), it is probable that the shunting caused by extracellular fluid trapped within this space is not reduced to the same extent in denervated as in normal muscle when demarcation potentials are measured. The increased membrane resistance which has been reported in denervated muscle (Nicholls, '56), would also tend to reduce the demarcation potential recorded in denervated muscle. These factors may account for the discrepancy between the results reported by Nicholls and here, and those reported by Bogatzki.

This paper also confirms Nicholls' ('56) finding that the amplitude of the AP of denervated frog sartorius muscle is unchanged.

Nicholls ('56) reported a significant reduction in the critical depolarization of denervated sartorius muscle, a finding which has not been confirmed by the present experiments. Examination of Nicholls' data indicates that in the process of measurement of the critical depolarization, the RP was reduced (due to the insertion of a second microelectrode) on the average by 11 mv in the case of the normal fibers, and by 7 mv in the case of the denervated fibers. In the present study this reduction in RP was avoided by the use of external stimulating electrodes. Jenerick ('56) has found in normal frog sartorius muscles that the CMP is independent of membrane potential for fibers with RP's in excess of -85 mv, but in fibers with low RP's ($<$ -80 mv), the CMP is not constant but varies directly with the membrane potential. The value reported by Nicholls for the CMP of denervated fibers (-47 mv) agrees well with that reported in the present study (-46.4 mv). His value for the normal fibers however (-38 mv) is lower than that reported here (-44.2 mv), and elsewhere: -45 mv (Fatt and Katz, '51); -46 mv (Nastuk, '53); -40 to -50 mv (Hagiwara and Watanabe, '55); -45 to

- 50 mv (Jenerick, '56). The discrepancy hus pertains only to the value for the CMP f normal muscle. The conclusion there- ore seems justified that the CMP of de- ervated frog muscle is unchanged, and his considered along with the fact that the P is unaltered, rules out the possibility hat a reduction in critical depolarization ontributes to the observed supersensitivity f denervated frog muscle.

Membrane potential measurements have een made in denervated mammalian mus- le, but conflicting results have been re- orted. Thus Ware, Bennett and McIntyre '54) and Lüllmann and Pracht ('57) found a progressive decline in the RP with ime after denervation, but Li, Shy and Wells ('57) reported that the RP was un- hanged after denervation. Kuschinsky, Lüllmann and Pracht ('59) reported that he AP produced by direct electrical stimu- ation was drastically altered in the diner- vated rat diaphragm (failure to overshoot, educed velocity of repolarization, develop- ment of a positive afterpotential), but Axelsson and Thesleff ('59) have reported hat in the denervated cat tenuissimus muscle, the CMP is unchanged, and the records of spikes shown (fig. 9) indicate hat the AP is otherwise unchanged. Cer- ainly none of the changes reported by Kuschinsky et al. ('59) for the denervated at diaphragm have been observed in the denervated frog sartorius muscle. In view of the unexplained contradictory results which have been reported for mammalian muscle, it is considered premature to re- ate the reported differences in reaction to denervation of frog sartorius and rat dia- phragm muscles to differences in the oc- currence of fibrillation in these two tissues.

Evidence has been presented (fig. 2 and table 3) for an increase in ACh sensitivity of the innervated muscles of operated frogs. Solandt, Partridge and Hunter ('43) have reported that innervated rat muscles undergoing a disuse atrophy, develop an increased ACh sensitivity. Recently Johns and Thesleff ('61) found some increase in ACh sensitivity of innervated muscles whose motor neurones had been isolated from all afferent input. One notable dif- ference observed between the operated and unoperated frogs used in the present study was that the operated frogs tended to be considerably less active in their bins in the laboratory (many operated animals seemed never to move at all, except in response to external provocation). Possibly, therefore the innervated muscles of the operated frogs in this study were essentially disused. If this is true then it would suggest that the sizeable reductions in the MRR and MRF of the AP's seen in both muscles of the operated frogs arose as a result of dis- use.

The estimates of the degree of supersen- sitivity of denervated frog sartorius muscle given by Kuffler ('43) and in this paper, while based on the application of ACh to a relatively small area of membrane in the vicinity of the endplate, agree generally with the estimates made by Nicholls ('56) and Miledi ('60), based on exposure of the entire muscle to ACh, although in all cases considerable variation from fiber to fiber was noted. This suggests that even though the entire membrane may become chemosensitive, the initial reaction of the fiber to diffusely applied ACh is still dom- inated by the area of greatest sensitivity in the vicinity of the endplate. In this connection it is worth noting that the range of supersensitivity observed in par- tially denervated fibers, while broad, is the same as that found for completely diner- vated fibers, although the size of the ACh sensitive membrane is smaller in the par- tially denervated fibers (Miledi, '60). The relatively diffuse methods of ACh applica- tion used by Kuffler and in this study would not distinguish between an increase of sensitivity of the endplate receptors and an increase of density of the ACh receptors in the extra-junctional membrane immedi- ately adjacent to the endplate. On the other hand, highly localized application of ACh by ionophoretic techniques has not revealed any significantly increased sen- sitivity of the endplate receptors (Axelsson and Thesleff, '59; Miledi, '60), although a several-fold increase would have been pre- dicted on the basis of a loss of ChE activity at the denervated endplates (but see Miledi, '61). The distribution of ChE activity in the vicinity of the normal sartorius end- plates is much more restricted in area than is the distribution of ACh sensitivity (Miledi, '61). Possibly therefore, the ACh sensitivity of the patch of endplate mem-

brane which posesses the highest sensitivity when innervated, has thus far escaped testing by the ionophoretic method after denervation. Until more data become available it will not be possible to state how much of a contribution is made to the observed supersensitivity of denervated skeletal muscle by an increased sensitivity of the endplate ACh receptors.

In any event it would seem that whatever factors participate in the production of supersensitivity, do so independently of the mechanism controlling the electrical excitability of the membrane.

SUMMARY

1. Resting potentials and action potentials (resulting from direct electrical stimulation) have been studied in single fibers of the chronically denervated sartorius muscle of the frog, using intracellular microelectrodes.

2. No difference was found between the two normal sartorii of unoperated frogs studied 4–24 hours apart with respect to resting potential, active membrane potential, critical membrane potential, maximum rate of rise and maximum rate of fall of the action potential, and the membrane potentials at the instants of attainment of these maxima.

3. Sartorii which had been denervated for 3–8 weeks also did not differ from contralateral innervated muscles of the same frogs with respect to any of the characteristics enumerated in 2.

4. The resting potential measured at the endplate site of the denervated muscle fiber did not differ from the resting potential of non-endplate membrane in the same fiber.

5. The acetylcholine sensitivity of individual denervated and innervated fibers was examined. A progressive increase in sensitivity developed so that at the end of 7 weeks, endplate regions of single fibers responded to concentrations of ACh 122 times less than those required by innervated fibers of the contralateral sartorius. Some increase in sensitivity to ACh was observed over this interval in the normally innervated contralateral fibers. Addition of eserine to the Ringer's solution bath resulted in a three-fold gain in sensitivity to ACh of innervated fibers, but little or no increase in sensitivity of the denervated fibers.

6. The resting potentials both of denervated and of contralateral innervated sartorii in operated frogs are smaller than the average resting potential of sartorius muscles of freshly received frogs.

7. The maximum rate of rise and the maximum rate of fall of the action potential are reduced by 30% and 40% respectively in both sartorii of operated frogs, below the values recorded in innervated sartorii of unoperated frogs maintained under the same conditions.

8. It is concluded that the structures which respond to chemical stimuli and those which are excited electrically may exist intermingled in the skeletal muscle membrane.

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Cortical Intracellular Synaptic Potentials

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Miniature endplate potentials recorded from skeletal muscle fibers were initially described in frogs (Fatt and Katz, '50) and subsequently in rats (Liley, '56; Li, '58), cats (Boyd and Martin, '55) and guinea pigs (Brooks, '56). These miniature potentials are thought to be generated by spontaneous liberation of quanta of transmitter substance from motor nerve endings (Fatt and Katz, '52; del Castillo and Katz, '54, '55). A synchronous occurrence of miniature endplate potentials represents a synchronous release of quanta of the transmitter. This gives rise to endplate potentials and spike discharges.

Recently, similar small potentials recorded from neurones in the cerebral cortex were reported (Li, '59). It was suggested that these small potentials recorded from the cortex are due to random bombardment of impulses through presynaptic fiber terminals and that the mechanisms of impulse transmission across the neuromuscular junction and in the cerebral cortex are probably the same. However, this investigation (Li, '59) was concerned with the "spontaneous" occurrence of small potentials and did not extend to a study of their responses to presynaptic stimulation.

In the present investigation evidence was sought for such small potentials elicited by presynaptic volleys. The experiments were carried out with intracellular electrodes recording from cat's somatosensory cortex while electrical stimulation was applied to the contralateral superficial radial nerve. It will be shown that the postsynaptic potentials thus produced consisted of many small potentials. Further, the postsynaptic potentials, which were evoked through polysynaptic pathways, were frequently found to persist for as long as 100 msec. These observations suggested the presence of a loose relationship between the prolonged postsynaptic potential and the negative wave in the response recorded from the surface of the cortex.

MATERIAL AND METHOD

Adult cats were used. They were anesthetized with an initial dose of intraperitoneal pento-barbitone sodium and subsequently with additional doses injected into the femoral vein through a cannula. The animals were immobilized in a stereotaxic instrument.

A superficial radial nerve was dissected, ligated, severed and bathed in warm mineral oil in a pool formed by the retracted skin edge. The central portion of the nerve in the oil was placed on two silver-silver chloride wires which served as stimulating electrodes.

The calvarium of the cat was opened and the cortex exposed. Movements of the cortex were minimized by opening the ventricle through the posterior fossa. In some cases the movement was further reduced by pressing the cortex with a small piece of "Plexiglas." In the center of the Plexiglas was a hole through which the recording micropipette electrode was inserted (Li, Ortiz-Galvin, Chou and Howard, '60). Stimulation of the nerve with square pulses of 0.01–0.50 msec. and 0.5–2.0 volts was applied once every 1–2 seconds. In some cases, stimulation was given in pairs. Responses to the stimulation were recorded with micropipette electrodes from the contralateral somatosensory cortex. In some cases, the change of potential at the tip of the microelectrode was recorded through a high-gain condenser-coupled amplifier with a time constant of 0.1 sec. and a low-gain direct-coupled amplifier. In others, the responses were

simultaneously recorded with a microelectrode and a surface electrode.

RESULTS

Penetration of cells in the cerebral cortex with the glass micropipette electrode was signalled by a sudden shift to negativity of the recorded zero potential. These changes were striking and offered reliable criteria for cell penetration. The negative steady potentials thus recorded measured between −30 and −100 mv.

It was frequently observed that large spike potentials (90 to 95 mv) were recorded with small steady potentials (−40 to −50 mv). Conversely, small spike potentials (30 to 50 mv) were sometimes recorded with large steady potentials (−60 to −100 mv). The small steady potentials and the small spike potentials suggested considerable damage of the cell membrane by the penetrating electrode, yet the accompanying large spike potentials and large steady potentials indicated an absence of serious injury to the cell. In some cases, the recorded steady potentials from apparently deteriorating neurones showed little change while the spike potentials gradually underwent fragmentation. There were also cases in which the level of the extracellular zero potential slowly declined and required frequent adjustment by applying backing voltage through the indifferent electrode. Thus, little confidence could be placed in the absolute values of the recorded steady potentials (or membrane resting potentials). This might have bee caused by "clogging" (del Castillo an Katz, '55) of the tip of the recording m cropipette electrode as the electrode move through the cortex. Since movement c the cortex with respect to the electrod could not be completely eliminated, "clog ging" of the electrode probably frequentl occurred, giving rise to a continuou change in "tip potentials" (del Castill and Katz, '55).

Of thousands of cells thus penetrate most died in a fraction of a second to few seconds or fired repeatedly at frequen cies of 30–100 per second, being una fected by nerve stimulation. There wer 34 cases in which the intracellular ele trode could be maintained for 2–40 min utes. In these cases nerve stimulatio evoked depolarization with or withou spike discharges. Cells with similar re sponses were found throughout the entir thickness of the cortex, although most c them were at a depth of about 0.8 mm below the surface, suggesting that thes cells were probably located at layer IV c the cortex (Li and Jasper, '53).

1. Spontaneous small potential an spike discharges. As previously reporte (Li, '59), spontaneous small potential were sometimes recorded with spike di charges. The records in figure 1A and are of the same cell taken at differen sweep speeds. Figure 1A shows sever individual small potentials, two small p tentials occurring in a step-wise manne and a potential with a much larger ampl

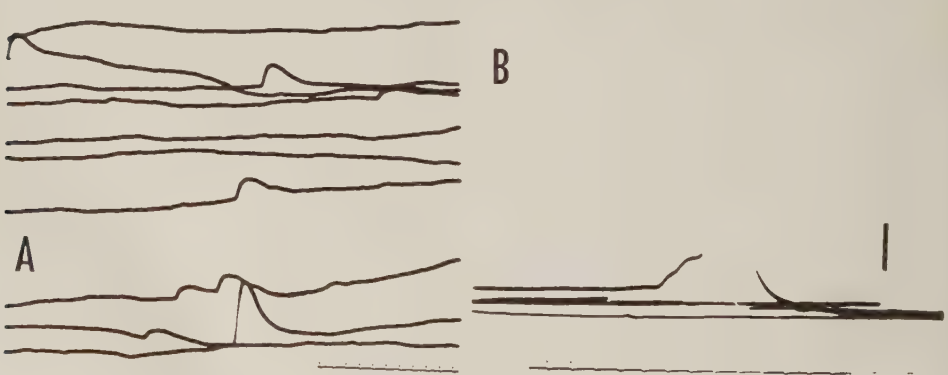

Fig. 1 "Spontaneous" small potentials (A) and spike discharges (B). Vertical bar represents 2 mv, time marks 1 msec. In this and subsequent figures, upward deflection recorded with microelectrodes represents positivity.

ıde. In figure 1B, steps are visible at the ɔot of the spike.

The frequency of the spontaneous small ɔtentials recorded from a given neurone aried, apparently corresponding to the vel of anesthesia and intensity of stimuıtion which the animal received. When ıe lightly anesthetized animal rubbed its mbs on the table, the small potentials hich had previously occurred at low freuencies often appeared in bursts of highrequency discharges. It was in the bursts ıat small potentials occurring in steps nd potentials having larger amplitudes ere seen. It was also in the bursts that pike discharges were occasionally oberved.

While there was a wide variation in the requency of small potentials recorded rom different neurones or from a same

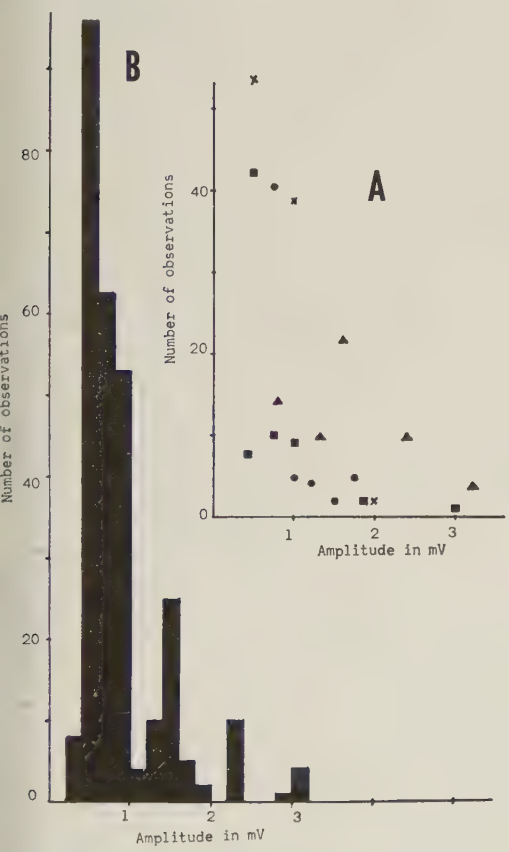

Fig. 2 A, amplitude measurements of "spontaneous" small potentials recorded from 4 cortical neurones. B, histogram from A.

neurone at different times, there was also variation in the amplitude of these potentials. Figure 1A shows 8 potentials having amplitudes of 1.0, 1.0, 0.5, 1.0, 0.5, 0.75, 0.5 and 2.75 mv respectively. The solid squares in figure 2A represent the amplitude measurements of 73 potentials recorded from this neurone. They appeared to distribute about a modal value of 0.5 mv. It is probable that the amplitude of an individual small potential, in this case, was 0.5 mv; and that the variation was due to either interaction or summation depending upon the interval between discharges (Fatt and Katz, '52). Figure 2B shows amplitude measurements recorded from 4 cells. It can be seen that the majority of the measurements fell between 0.5 and 1.0 mv and that the elevations in the histogram suggest a temporal summation of two or three small potentials. It is to be noted that although the amplitudes of small potentials varied, the durations were fairly constant, ranging between 4 and 6 msec. (fig. 1).

2. *Separation of response components by paired synaptic volleys.* Upon the arrival of a synchronous presynaptic volley, the intracellular microelectrode recorded a depolarizing potential (or synaptic potential) leading to a spike discharge. The spike potential developed in two stages, similar to the spike response recorded from spinal motor neurones (Brock, Coombs and Eccles, '52; Fatt, '57; Fuortes, Frank and Becker, '57). Thus, it could also be assumed that the discharge of a cortical neurone involves successive invasion of at least two "parts" of the neurone.

As in experiments of the spinal cord, invasion of a cortical neurone could be forestalled by applying a preceding volley at appropriate intervals. Figure 3A shows the two components of the spike to be separated when the second stimulus was applied 44 msec. after a preceding stimulus. At this interval the second stimulus evoked a small spike response having an amplitude of 17 mv. This suggests that there was a blockage between the two "parts" of the cell generating the two components of the spike. A further decrease in the interval between the two stimuli eliminated the spike completely,

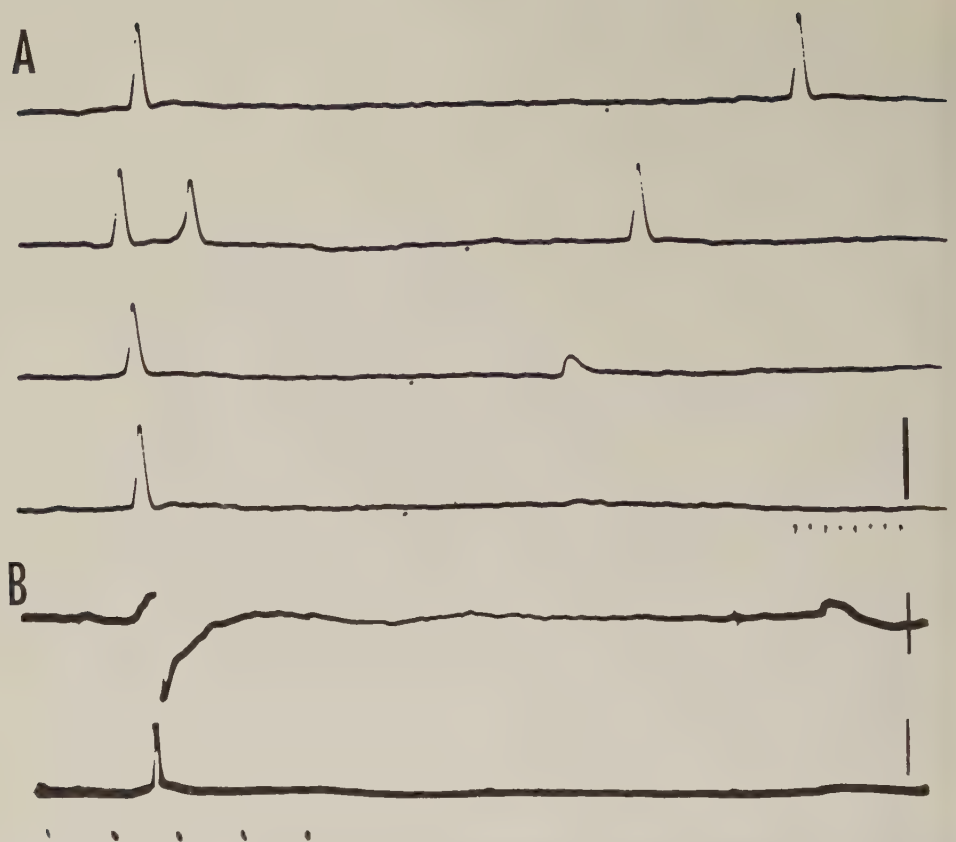

Fig. 3 Intracellular responses of two cortical sensory neurones to paired stimuli applied to the nerve. A, intervals between two stimuli: 57, 47, 44 and 43.6 msec. Vertical bar represents 50 mv; time marks 1 msec. B, interval between two stimuli: 100 msec. Vertical bars represent 10 mv for the top tracing obtained with a condenser-coupled amplifier and 50 mv for the bottom tracing obtained with a direct-coupled amplifier. The time marks 10 msec.

but elicited ripples of potential which occurred where the spike response had been anticipated. In the example shown in figure 3B, the first stimulus evoked a depolarizing potential (or synaptic potential) which, having surpassed a level of 5 mv, set off a spike discharge. The depolarizing potential occurred in stages: thus, in its rising phase there was a notch at a level of 2.7 mv (see first line of figure 3B, recorded with a high-gain condenser-coupled amplifier). When the second stimulus was applied 100 msec. after the first stimulus, only depolarization of 2.7 mv and 13 msec. was evoked.

It should be noted that the "recovery interval" of a neurone following a synaptic volley (47 msec. for the response to the second stimulus in the second record c figure 3A) was not due to the refractor ness of the cell itself. The cell was capabl of repetitive firing within an interval c 4 or 5 msec. (as shown in the response t the first stimulus in the second record c figure 3A), although the spike potential occurring at this interval were smaller i amplitude, longer in duration and of more prominent inflection in the risin phase.

The "recovery intervals" thus recorde intracellularly from 7 cells were 47, 5 62, 77, 90, 92 and 110 msec. Prolonge and variable "recovery intervals" were als recorded from single cells with extracellu lar electrodes (fig. 4) and from the surfac of the somatosensory cortex (Marshal

Woolsey and Bard, '41; Marshall, '41). This was apparently due to the effect of barbiturate anesthesia as demonstrated by Marshall, Woolsey and Bard ('41).

Figure 4 serves to illustrate in addition that by the recording with extracellular electrodes the presence of unitary small potentials in a synaptic potential was also

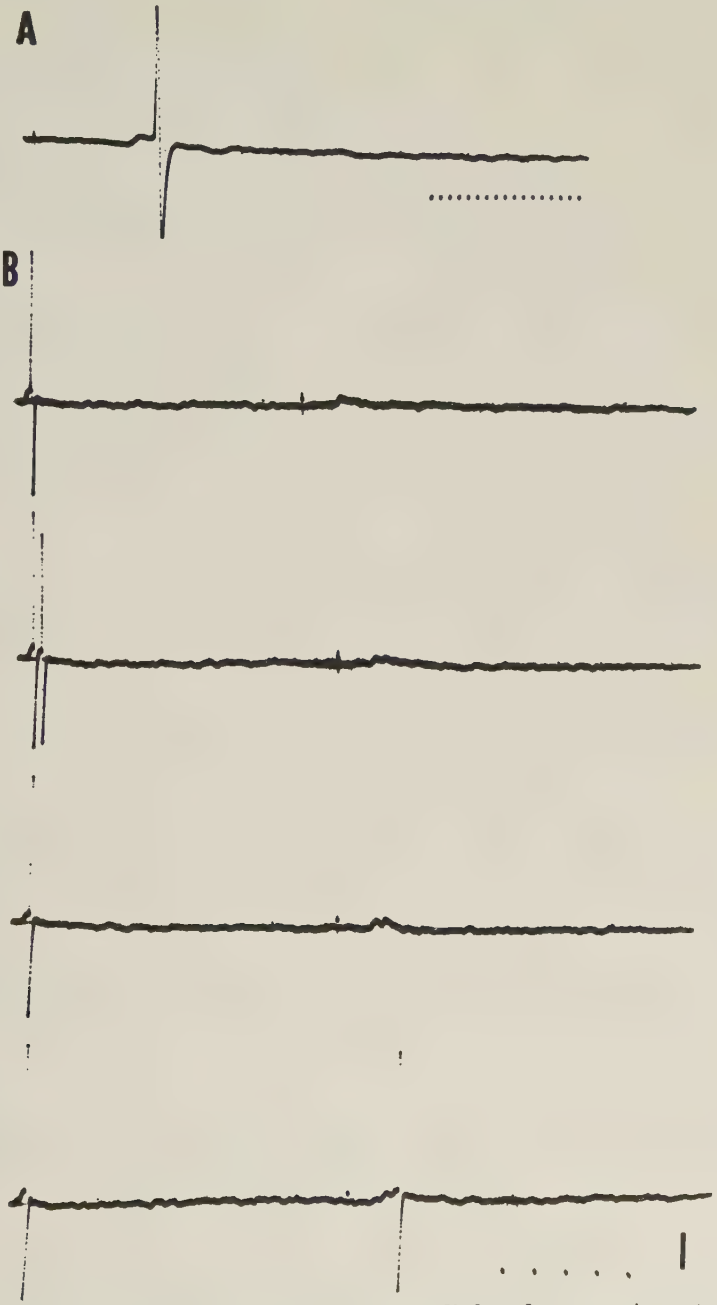

Fig. 4 Extracellular responses to paired stimuli applied to the nerve. A, control response. B, intervals between two stimuli: 93, 98, 98.5 and 101 msec. Vertical bar represents 2 mv. The time marks 1 msec. in A and 10 msec. in B.

suggested. The spike discharge shown in figure 4A was preceded by a prepotential having an amplitude of 0.57 mv. When a second stimulus was applied 93, 98.0 and 98.5 msec. after a preceding stimulus, only prepotentials were recorded. The prepotentials, when occurring in the absence of spike discharge, were no larger than 0.50 mv and appeared to consist of several small components. When the second stimulus was applied 101 msec. after a preceding stimulus, the prepotential at the foot of the spike discharge occurred in a step-wise fashion (bottom record in fig. 4).

In two cases, the intracellular spike potential gradually deteriorated; and upon stimulation of the nerve, slow potentials were elicited. When stimulation was applied after a preceding synaptic volley, the slow potential occurred in a step-wise manner, suggesting that the slow potential represented the sum of numerous small potentials. This is illustrated by the re cordings in figures 5, 6 and 7.

In figure 5A, spike discharges were re corded shortly after the electrode entere the cell; in B–G, they were recorded a various intervals after A. It can be see that there was a gradual separation of th first and second spike components. Du ing this period (fig. 5A–G) the interspik intervals showed little change and mea ured between 12 and 88 msec. with a average frequency of 25 per second. In G only the first spike component remaine with an amplitude of 21 mv and duratio of 2.5 msec.

The responses in figure 6 were obtaine from the same cell shown in figure 5. Th top recording (fig. 6) shows a respons consisting of a small spike discharge an a prolonged depolarization potential. Th small spike potential measured 18 mv i amplitude and 2.5 msec. in duration. Th

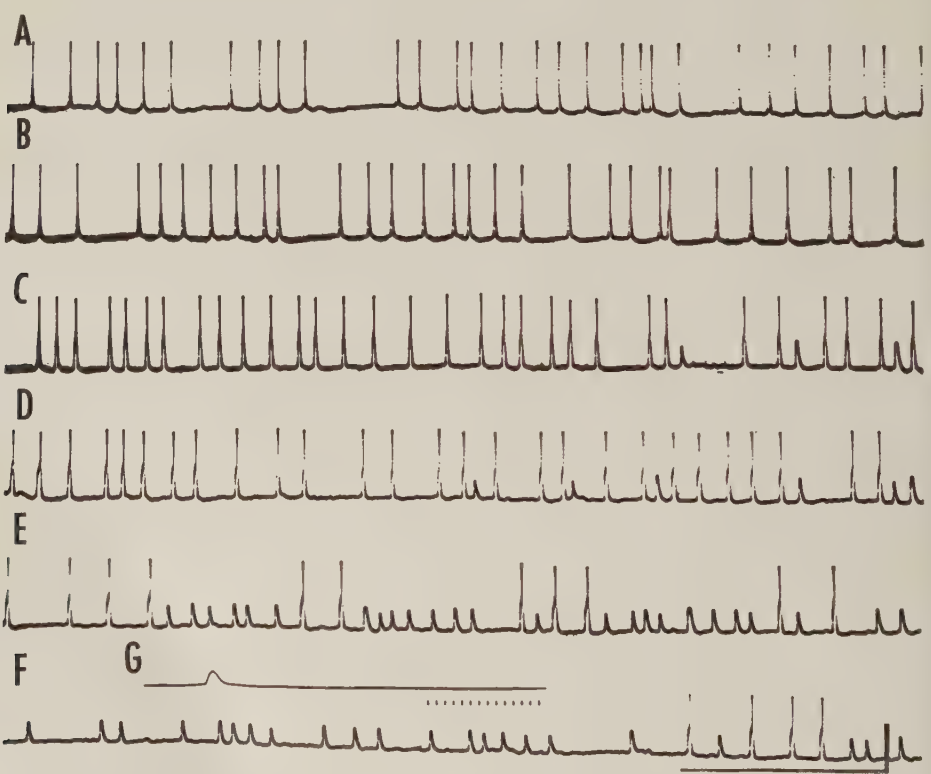

Fig. 5 Gradual separation of spike components. A, recorded shortly after electrode entered the cell; B, C, D, E, F and G, recorded at various intervals after A. Vertical bar represents 50 mv; horizontal bar, 200 msec. In G, the time marks 1 msec.

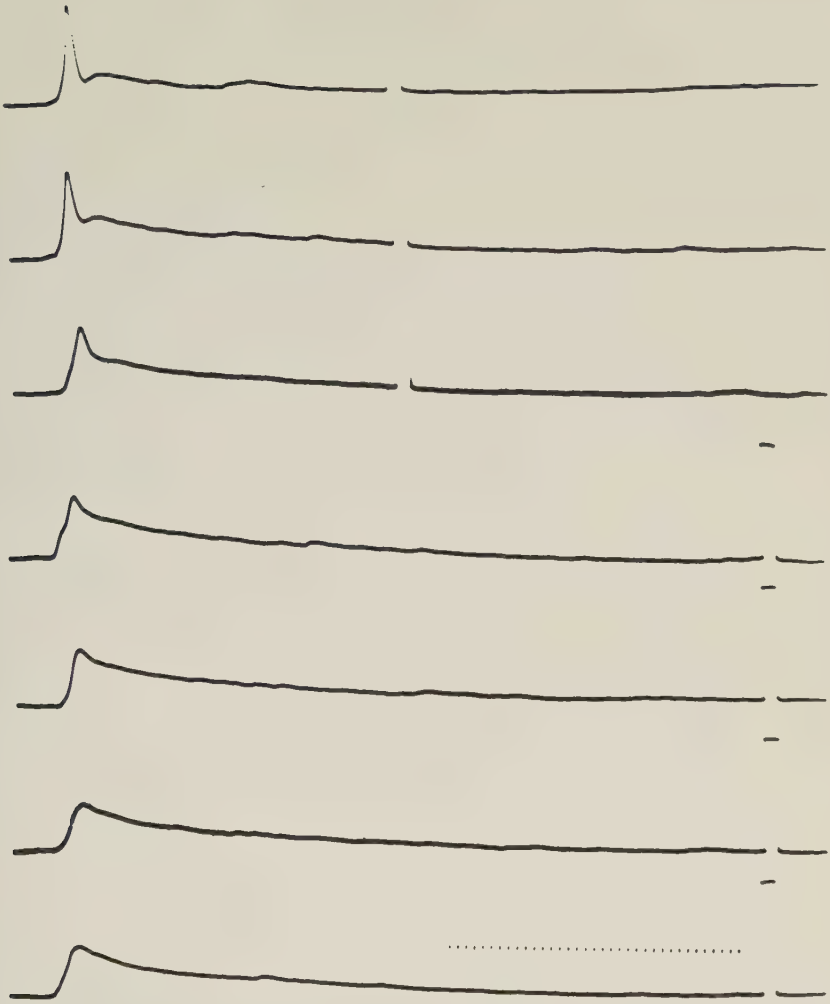

Fig. 6 Gradual disappearance of first spike component in response to nerve stimulation. Square pulses at the end of last three sweeps represent 20 mv. The time marks 1 msec.

ottom recording (fig. 6) shows only depolarization potential in the response. Depolarization rose to a peak of 9 mv in 3 msec., then slowly subsided in about 30 msec.

Figure 7 consists of responses to paired stimuli recorded from the same cell after the responses in figure 6 were obtained. The response to the first stimulus was a simple elevation of potential with occasional small potentials in the falling phase (6th record in fig. 7). On the other hand, the responses to the second stimulus, applied at intervals between 75 and 135 msec., consisted of a series of small potentials occurring in steps. A gradual shortening in the stimulus intervals from 135 msec. to 75 msec. (fig. 7, 7th to first record) was accompanied by a progressive decrease in the number of small potentials and in the size of the resulting slow potentials. With further shortening of the stimulus interval, no small potentials or slow potentials were produced by the second stimulus. This would indicate that the occurrence of small discharges, which could be synchronously evoked, ceased to be synchronous following a preceding synaptic volley applied at appropriate intervals. When the preceding synaptic volley was applied at short enough intervals, the discharge of small potentials was suppressed.

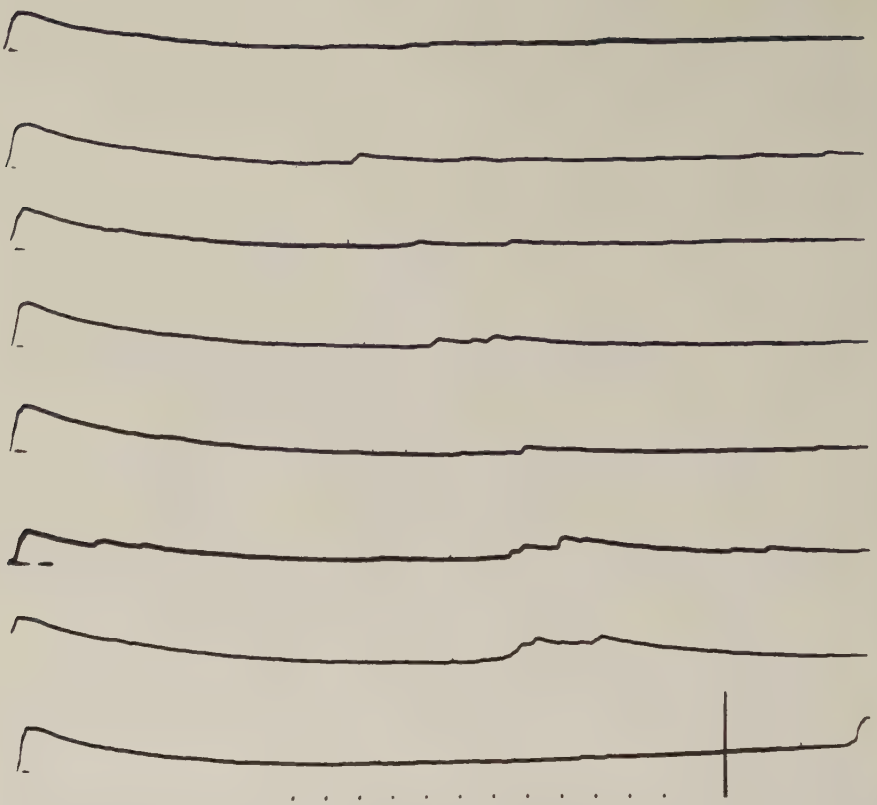

Fig. 7 Depolarization elicited by paired stimuli applied to the nerve. Vertical bar represents 20 mv; time marks 10 msec.

3. *Summation of small potentials leading to spike discharges evoked by synaptic volleys.* In 6 experiments, small potentials were elicited by nerve volleys having a strength insufficient to produce spike responses. When the stimulus strength was increased, summation of small potentials resulted. A further increase in the stimulus strength gave rise to spike discharges. Figure 8 shows the responses of a neurone recorded through a high-gain condenser-coupled amplifier (first line of each record) and a low-gain direct-coupled amplifier (second line of each record). In A, two small potentials of identical size and shape occurred 20 msec. following a weak stimulus applied to the nerve. B shows that a stronger stimulation evoked a response with a latency of 10.5 msec. In the response there were a notch in the rising phase and two small potentials in the falling phase. A further increase in the stimulus strength gave rise to responses
higher amplitude (fig. 8C) and spike di
charges (fig. 8D and E). The latencies
spike discharges were shortened when th
stimulus strength was increased, but th
latencies of the responses showed no si
nificant change (fig. 8, B, C, D and E).

4. *Prolonged depolarization potentia
and surface responses.* In 4 experiment
success was achieved in recording the i
tracellular potentials simultaneously wi
the surface responses to nerve stimulatio
Failures were caused by the fact that whe
cortical movements were minimized
pressure with the Plexiglas, responses r
corded from the surface were either t
small or absent. In these 4 experimen
in which no pressure was applied to th
cortex, the intracellular recording laste
for about a minute. Nevertheless, the
brief observations suggested that there w
a loose relation between the surface-neg

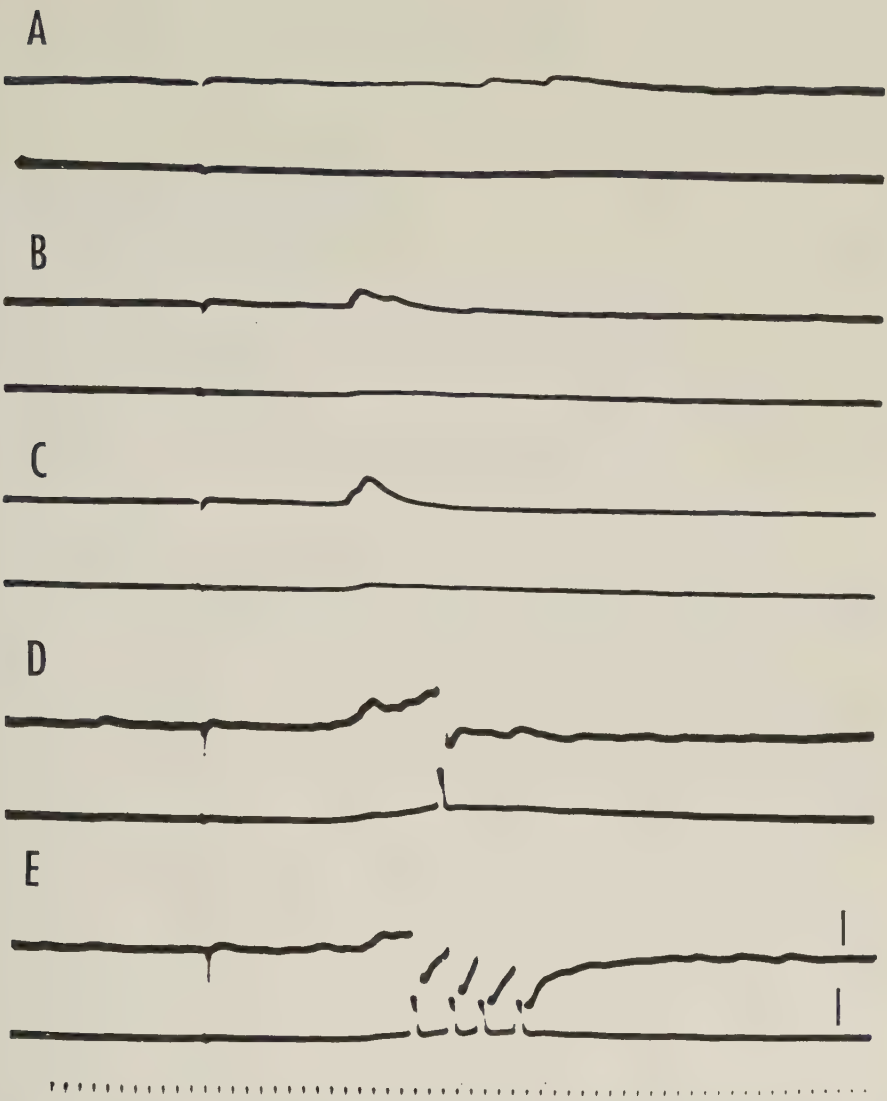

Fig. 8 Responses to synaptic volleys of gradually increasing stimulus strength. First line of each record was obtained with a high-gain condenser-coupled amplifier and the second line with a low-gain direct-coupled amplifier. Vertical bars represent 10 and 50 mv; time marks 1 msec.

ve potentials and the intracellular slow otentials.

Figure 9 presents records of responses otained before and after the microelecode entered a cell. The strength of stimation used to evoke the responses in B, D, E and F was gradually reduced. In D and E two parallel lines were drawn, ttending from the levels of potential reorded before application of the stimulus.

In this experiment nerve stimulation repeatedly evoked a surface response and an extracellular spike discharge (fig. 9A). The surface response had an average latency of 6 msec. and consisted of a surface-positive potential, which reached its peak in 6.5 msec. and gradually fell into a surface-negative wave. The negative wave of potential lasted for 40 msec. or longer. The response recorded with the

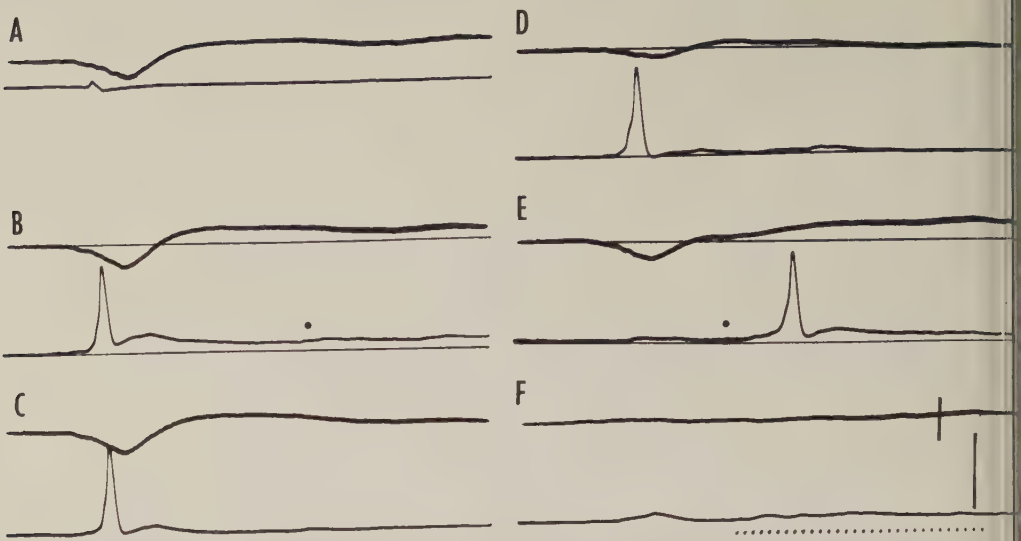

Fig. 9 Responses to synaptic volleys recorded simultaneously with a surface electrode and a microelectrode. A, before and B to F, after the microelectrode entered a cell. B to F are responses to stimulation of gradually decreased stimulus strength. Vertical bars represent 1 and 50 mv. The time marks 1 msec. For recordings from the surface, positivity is downwards.

extracellular microelectrode (fig. 9A) had a latency of 7 msec., and was composed of a positive-negative spike with a total duration of 4 msec. There was no significant change of potential recorded before or after the spike response. With a shift to negativity of the recorded potential to −53 mv (fig. 9B), an intracellular spike response was recorded. The spike potential was not followed by afterpositivity. Although the measurement of −53 mv might not represent the true value of the membrane potential of the cell, the absence of afterpositivity following the spike potential suggested that the membrane potential of the cell was probably not reduced (Eyzaguirre and Kuffler, '55). The spike potential (fig. 9B) was preceded by a synaptic potential and followed by a prolonged depolarization potential. The prolonged depolarization potential was not smooth but wormy in appearance; and appeared to run a parallel course with the surface-negative potential (fig. 9, B, D and E). Occasionally, small steps of potential (as indicated by a dot in figure 9B) could be seen. When a weak stimulus was applied to the nerve (fig. 9E), spike discharge with similar latencies did not occur. Instead, there was a small synapt potential which rose and fell, and agai rose (indicated by a dot in figure 9E) reach its firing level of 5 mv. With th occurrence of the spike discharge the was a simultaneous growth of the surfac negative potential. When a stimulus subthreshold strength was applied to th nerve, only small disturbances of potenti were recorded from the neurone and n significant change of potential was r corded from the surface (fig. 9F).

Figures 10A, B and C are the othe examples showing that the evoked slo potentials recorded with the intracellula electrode persisted approximately as lon as the negative potential recorded fro the surface. In the case shown in figu 10C, pair stimuli were applied. When th surface-negative potential in the secon response became smaller than that in th first response, the intracellular slow po tential (or synaptic potential) occurred i steps (fig. 10C).

Examination of the responses of all th 34 cells revealed that prolonged depolar zation in the response was recorded fro 27 cells.

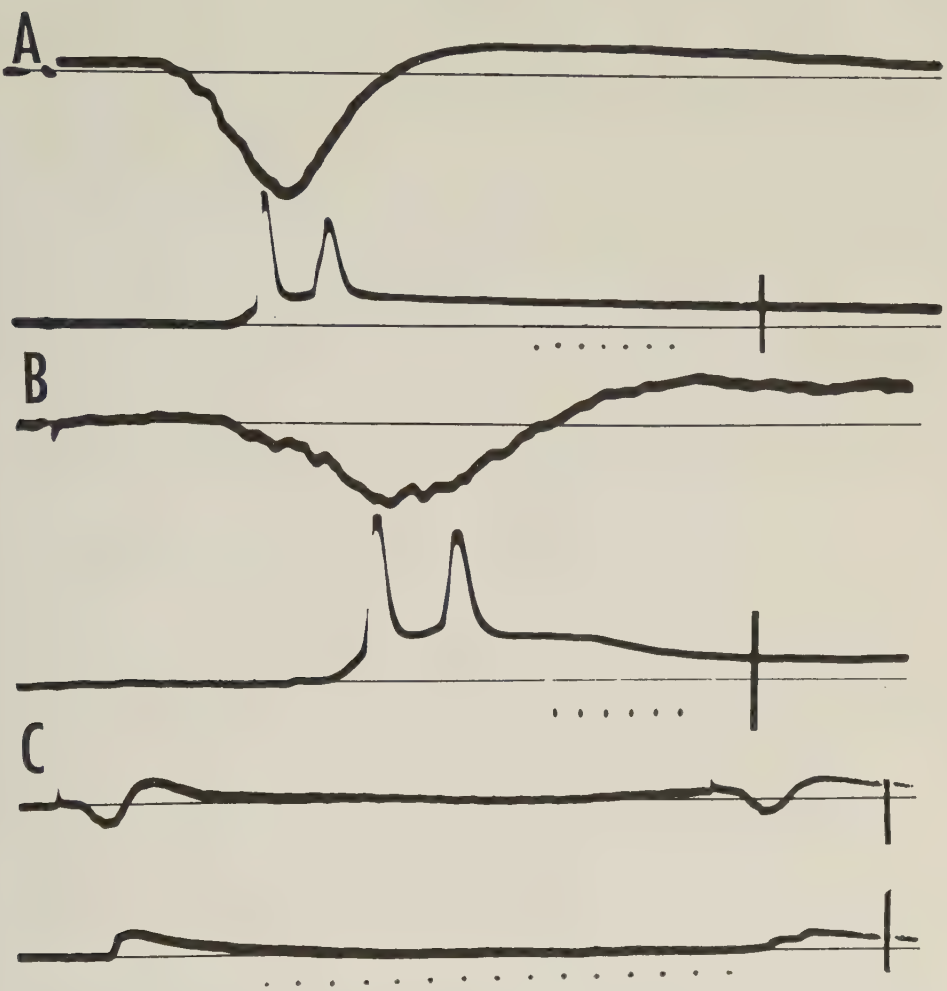

Fig. 10 Responses simultaneously recorded from surface and three cells. Note that in C, the responses to the second of the paired stimuli show a relatively small surface-negative potential and an intracellular synaptic potential occurring in steps. Vertical bars represent 50 mv in A and B; 2 mv and 20 mv in C. The time marks 1 msec.

A typical response recorded from the ortical surface and responses recorded om the 34 cells to stimuli of supramax- nal strength were enlarged to a same me scale. The responses were grouped nd traced according to their response la- ncies. These are shown in figure 11. can be seen that in 27 cases, prolonged epolarization occurred, lasting approxi- ately the same length of time as did ie surface-negative potential following e primary response (S). In 4 of the cases (cell nos. 11, 19, 20 and 26), ly slow depolarization potential was elic- ited. In 4 cases (cell nos. 17, 25, 29 and 33), the duration of depolarization poten- tials was relatively shorter, running be- tween 10 and 20 msec. In three cases (cell nos. 18, 27 and 34), spike discharges were followed by positive after-potentials. In 4 cases (cell nos. 15, 16, 23 and 24), the latency of spike discharges was much longer than the latency of depolarization potentials, with a discrepancy as much as 14 msec. The latency of the responses recorded from 34 cells ranged from 7 to 17 msec., most fell between 7 and 10.5 msec. (cell nos. 1 to 29).

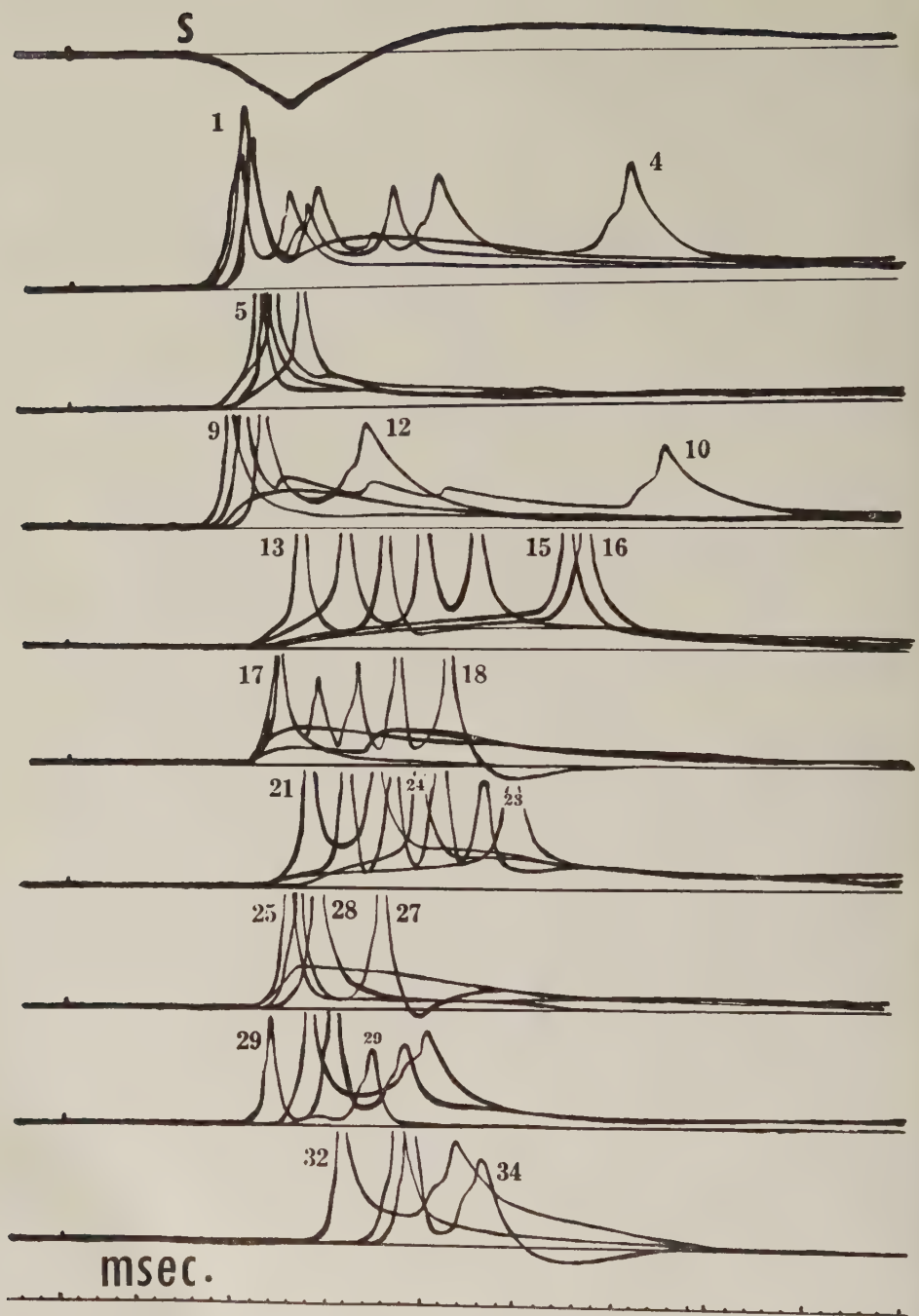

Fig. 11 Tracings of a typical surface response S and intracellular responses recorded from 34 cells (cell nos. 1 to 34). The responses were enlarged to a same time scale and grouped according to their latencies.

DISCUSSION

Spike potentials with two components ere assumed to have been recorded from ll bodies of spinal motor neurones Brock, Coombs and Eccles, '52; Fatt, 7; Fuortes, Frank and Becker, '57). Like- se, in the present study, the spikes hich developed in stages can be assumed have been recorded from the cell bodies cortical neurones.

After the intracellular microelectrode as positioned, the cortical neurone began deteriorate (Li, '55) but continued to spond to external stimuli for as long as –40 minutes. Deterioration of spike po- ntials occurred in two stages: the second ike component was shed, and then the st component. Since the tip of the mi- oelectrode was assumed to be in the ll body, deterioration probably occurred ere before involving other parts of the ll. This suggests that the activity of the ll body is responsible or at least neces- ry for the presence of the second com- nent in a spike discharge. This is in eping with the notion proposed by Brock, oombs and Eccles ('53) and Fuortes, ank and Becker ('57) that the second mponent of the spike is generated by the madendritic portion of the cell. It is to noted that two-component spikes were so recorded from deteriorating axons and eletal muscle fibers; though in these ses, the ratio of the two components was riable and the deteriorating process was ief, lasting for no longer than a few conds.

From neurones which had failed to pro- ce spike discharges, slow potentials were cited. These slow potentials could be oken down into small potentials by ired stimuli, if applied at short enough tervals.

Similar slow potentials were also elic- d from cells before the cells were seri- sly injured by the penetrating electrode. ne slow potentials thus elicited were und to be the sum of many small poten- ls; and spike discharges resulted when e summed potential reached a certain e. The slow potentials were not affected spike discharges and there were small tentials in the falling phase.

Considering that the impulses initiated in the peripheral sensory nerve must go through several relay stations before arriving at the cortex and that the diameters and conduction velocities of the nerve fibers are not the same, a prolonged synaptic bombardment of cortical neurones could be expected. The arrival of these impulses at different times might have been the cause for which random small potentials from the postsynaptic neurones were produced.

It was reported by Coombs, Eccles and Fatt ('55) that the synaptic potential recorded from spinal motor neurones was abolished by the second component of the spike discharge. They assumed that complete invasion of the neurone occurred when there was a full-sized spike discharge. In the present study on responses of cortical neurones, such abolition was seldom observed. This is probably due to the presence of the relatively large apical dendrites and the wide distribution of synaptic terminals in the cerebral cortex. Although synaptic terminals are known to be concentrated in large numbers on the cell body and at the distal portion of the apical dendrite (Chang, '52), they are probably also found on the surface of dendritic branches. It is possible that a large part of synaptic activity takes place in the dendrites (Eccles, '51; Li, Cullen and Jasper, '56; Fatt, '57; Bishop, '58; Grundfest, '58) and creates an electrotonus or synaptic potential which is, however, insufficient to set off spike discharges. It is also likely that, when a spike discharge occurs, invasion of the cortical neurone does not necessarily spread to involve the entire length of the apical dendrite and all of the dendritic branches. Thus, electrotonic potentials from dendrites were recorded simultaneously with spike potentials. Electrotonic potentials (synaptic potentials) alone were elicited from neurones incapable of producing spike discharges.

SUMMARY

Recordings with intracellular glass micropipette electrodes from the somatosensory cortex in the cat showed "spontaneous" random small potentials. These

small potentials were further investigated upon stimulation of the contra-lateral radial nerve. The results and conclusions were as follows.

1. The rubbing of the animal's limbs on the table caused "spontaneous" small potentials to fire at high-frequencies, and, sometimes, gave rise to spike discharges. Steps were frequently recorded at the foot of the spikes. Amplitude measurements of 302 small potentials recorded from 4 cells showed that they distributed about a modal value of 0.5 mv. Their duration ranged between 4 and 6 msec.

2. Stimulation of the nerve by gradually increasing the stimulus strength caused summation of small potentials leading to spike discharges.

3. The foregoing suggest that the small potentials were unitary synaptic potentials or miniature excitatory postsynaptic potentials (miniature e.p.s.p.) similar to miniature end-plate potentials recorded from neuromuscular junctions.

4. Fragmentation of the response by paired stimuli applied to the nerve revealed two components in the spike potential and a synaptic potential.

5. Recording from deteriorating neurones showed that the second component of the spike potential was shed, then the first component. Since the spike potential was assumed to be recorded from nerve cell bodies, deterioration probably occurred there first before involving other parts of the neurone. Thus, the cell body might have been responsible or at least necessary for the presence of the second component of the spike.

6. Neurones which failed to generate the two components of the spike discharge would respond to synaptic volleys with prolonged depolarization comparable to synaptic potentials. These potentials probably represent electrotonic potentials created in dendrites by synaptic impulses.

7. Prolonged depolarization, which was not affected by spike discharges, was found in responses of 27 among the 34 cells under investigation. The prolonged depolarization lasted approximately the same length of time as did the surface-negative potential in the response recorded from the cortical surface.

8. Brief observations on simultaneou recordings from cortical surface and co tical neurones showed that the surfac negative potential grew with depolariz tion of the cell.

9. Latency studies from responses of 3 neurones showed a range, in most case between 7 and 10.5 msec.

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he Effect of Cooling on Whole Hearts in Culture

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Although modern "open heart" surgical echniques have provoked considerable interest in the effects of cold stress on cardiac tissue, we have been unable to find ny record of an attempt to investigate ne problem using the large mass of data nat can be accumulated from tissue culure explants with modern automatic recording techniques. The study to be reorted concerns the effect of repeated ooling on the electrical activity of 31 hole chick embryo hearts isolated from ne other tissues of the body and from ach other as explants in tissue culture. sing automatic electronic monitoring and ecording equipment, a large body of data 13,000 observations) was accumulated n their behavior over a relatively short eriod of cooling. Each heart was kept nder identical conditions. It was exlanted from a 7-day chick embryo onto 36-gauge bare platinum electrode in the ngle of an L-shaped piece of cellulose ponge on a coverglass. This was placed n a culture tube and a flow system allowed upernatant to flow at 1 ml/day and is escribed together with the culture techique, chamber and supernatant in previus publications (Estborn et al., '58; Cuningham and Herbst, '60). The chamber nd flow system were clipped to a metal rame and the electrodes connected to a hielded cable by means of insulated bindng posts. The whole was placed in an ncubator at 37°C.

The temperature experiments were tarted when the hearts had been 72 hours n culture, since our previous experience ndicated that heart tissue in culture eaches a plateau of functional activity y this time (Cunningham and Estborn, 60; Estborn and Cunningham, '61).

The culture incubators were specially esigned to maintain a uniform temperaure. Although the temperature in the culture chambers followed that in the incubator, the insulating effect of the glass, air and fluid in the culture chamber and flow system made any change in temperature in the heart tissue lag behind that in the incubator by about 15 minutes. Since we were interested in the form of the change in the functional activity of heart tissue in response to cooling, it was decided to use changes in the incubator temperature (which needed only single measurements) as an indication of what was happening to the cultures. The technical ease of doing this outweighed the increased accuracy but greater technical difficulty of using the average of 31 separate temperature readings taken simultaneously inside the culture chambers. Another consideration was the relatively small size of the 15 minutes lag in temperature change compared to the total length of the experiment. The temperature reduction used in these experiments was from 37°C (normal incubator temperature) to between 21 and 23°C. This was effected by inactivating the heating system of the incubators and opening their doors. The subsequent rewarming was achieved by closing the incubator doors and reactivating the heating system till the desired rise in temperature was obtained. The actual incubator temperature in each experiment is given in degrees centigrade as a solid line in the upper part of figures 2, 3 and 4. The cycle of temperature change was repeated thrice (figs. 2, 3 and 4) using the same group of cultures. The intervals between starting time of cycle one and two was 8 hours and between two and three was 18 hours.

Of the 31 whole hearts used in these experiments, the average number showing electrical activity during any one 20-second survey was 21 (29 individual checks were made for electrical activity in each

culture in the series). Since the mean deviation from this average of 21 was less than 5%, the total rate and potential were used in place of average rates and average potentials. The cultures were under the constant surveillance of an automatic electronic monitoring and recording device which measured and registered the amplitude and frequency of all electrical potentials from all hearts used in the study. Every 20 seconds it recorded separately on a paper strip recorder straight lines whose lengths were proportionate to the logarithms of the total rate and total potential for all active hearts for the first 15 of these 20 seconds. Figure 1 shows the actual record of the results of the second cycle of temperature change. Each vertical line in the lower record represents logarithmically the total potential and each in the upper record, the total rate from all hearts in the first 15 of the previous 20 seconds. Since the positioning of the electrodes and method of collecting, amplifying and recording these potentials is unchanged during the whole experiment, each value for potential rate is strictly comparable with all other similar values. Although 15 second observations were made on all active hearts each 20 seconds for the whole duration of the experiments, only the figures from one such 15 second period every three minutes were used for the purposes of this communication.

Figures 2, 3 and 4 give the results of the experiments. Since we are interested in the form of the change in functional behavior rather than in absolute measurements, the variables on the Y-axis (total rate, total potential and potential per beat) are expressed as a percentage of the average of all such measurements made during the experiment. The total rate is given as a solid line and the total potential as a broken line in the left-hand part of each figure, while the value for the potential per beat (obtained by dividing the total potential by the total rate for the same instant) is given as a solid line in the right-hand part of the figure.

The general response of the heart tissue to change of temperature was similar on each occasion— the contractile rate an total potential dropped and rose with th

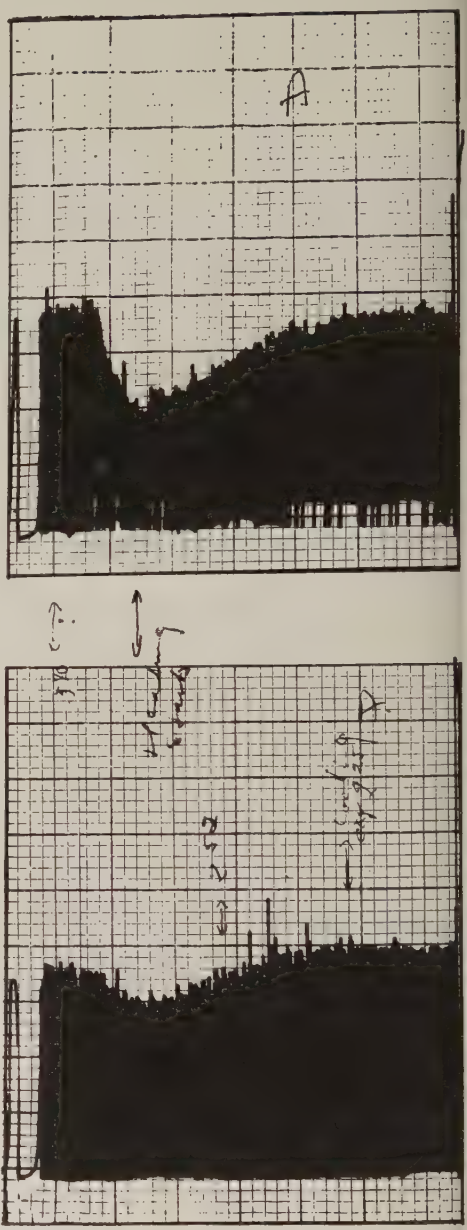

Fig. 1 Part of the actual record of the exper ment. Each vertical line in the upper trace re resents the logarithm of the total rate from a cultures for 15 of the previous 20 seconds. Eac vertical line in the lower trace represents the log rithm of the total potential from all cultures f 15 of the previous 20 seconds. Time (on the X axis): each major division represents 40 second

mperature. *The contractile rate* fell most idly during the first cooling episode en a drop of 13°C in approximately o hours caused a rate decrease of % —about one-and-a-half times the de- ease of 19% in response to a tempera- re drop of 13.5°C in approximately the me time in the second cooling episode. e third cooling episode caused a de- ease of 16% in response to a tempera- re drop of 12°C in 100 minutes. The oling process varied a little in extent d duration on each occasion, but it used a drop in rate to nearly 87% all ree times.

The rewarming caused the expected rate crease which seemed to be facilitated by e repetition of the cooling and warming. n the first occasion, the rate took 45 min- es to return from its lowest level to the e-cooling rate (the incubator took 75 inutes). On the second occasion, the te took 36 minutes to return from its west level to its pre-cooling level though the incubator took 80 minutes. On the third occasion, the rate took 30 minutes from its lowest level to its pre-cooling level while the incubator took 60 minutes. It is possible that the lessened rate decrease with repeated cooling and the greater rate increase with repeated rewarming were due to the excitatory effect of the accumulation of breakdown products from heart tissue killed by repeated temperature change. Previous experience with hearts in culture (Cunningham and Estborn, '60; Estborn and Cunningham, '61) has shown a similar increase in contractile rate during the initial 72 hours in culture which has been attributed to breakdown products from heart tissue damaged during explantation. However, these alterations in rate response and perhaps even the accumulation of breakdown products, may have been due to fatigue. This is supported by the fact that these changes were most marked between the first and the second cooling episodes when

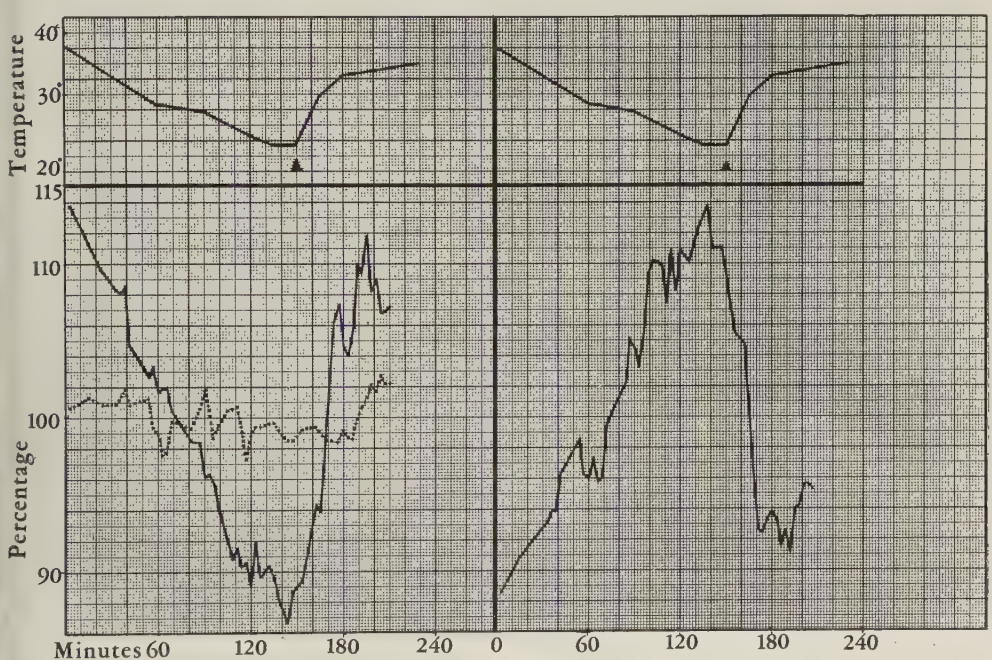

Fig. 2 X-axis, time; each minor division represents three minutes. Y-axis: upper left and right; incubator temperature in degrees Centigrade. Pointer indicates where reheating was commenced. Lower left; solid line—total rate for all 21 cultures expressed as a percentage of the average total rate for the whole episode. Broken line—total potential for all 21 cultures expressed as a percentage of the average total potential for the whole episode. Lower right; potential per beat expressed as a percentage of the average potential per beat for the whole episode.

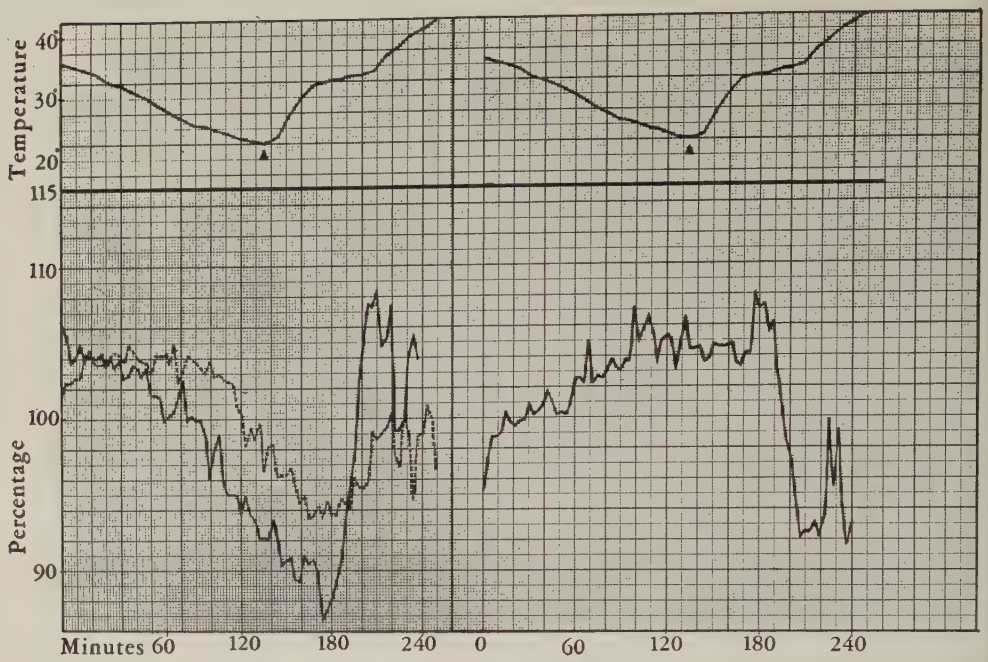

Fig. 3 X-axis, time; each minor division represents three minutes. Y-axis: upper left and right; incubator temperature in degrees Centigrade. Pointer indicates where reheating was commenced. Lower left; solid line—total rate for all 21 cultures expressed as a percentage of the average total rate for the whole episode. Broken line—total potential for all 21 cultures expressed as a percentage of the average total potential for the whole episode. Lower right; potential per beat expressed as a percentage of the average potential per beat for the whole episode.

the rest period was only one-and-a-half hours while, after a rest of 17 hours between the second and the third episodes, the response was more like that noted during the first episode.

The total potential shows a relatively small decrease during the first cooling episode—a drop of only 4% as compared with a drop of 11% on the second occasion and less than 8% on the third occasion. The response to cooling took about the same time on all occasions. The return of the potential to its pre-cooling level took 40 minutes on the first occasion, had not been completed in 75 minutes on the second occasion and took 45 minutes on the third occasion. This particular behavior of the potential was the opposite of that which we have previously postulated as being due to breakdown products (Cunningham and Estborn, '60; Estborn and Cunningham, '61). Thus, it is more likely that the change in behavior of both the rate and potential with repetition of the cooling and reheating process we fatigue phenomena and not due to the a cumulation of breakdown products.

The behavior of the values for *the p tential per contraction* are probably th most interesting of the measurements. I each case there was a progressive i crease in the potential per contractio with decreased temperature which pro ably started as soon as the insulation the chambers allowed any temperatu variation in the culture itself. This chan was greatest during the first episode cooling when it increased 28% as co pared with an increase of 13% on th second occasion and 18% on the thir occasion. The potential per contractio dropped sharply as soon as the temper ture in the tubes began to increase (save the second occasion of rewarming whe there was a delay of nearly an hour in th response). There was a decrease of 20 in 30 minutes of reheating on the first o casion, a decrease of 15% in 73 minut

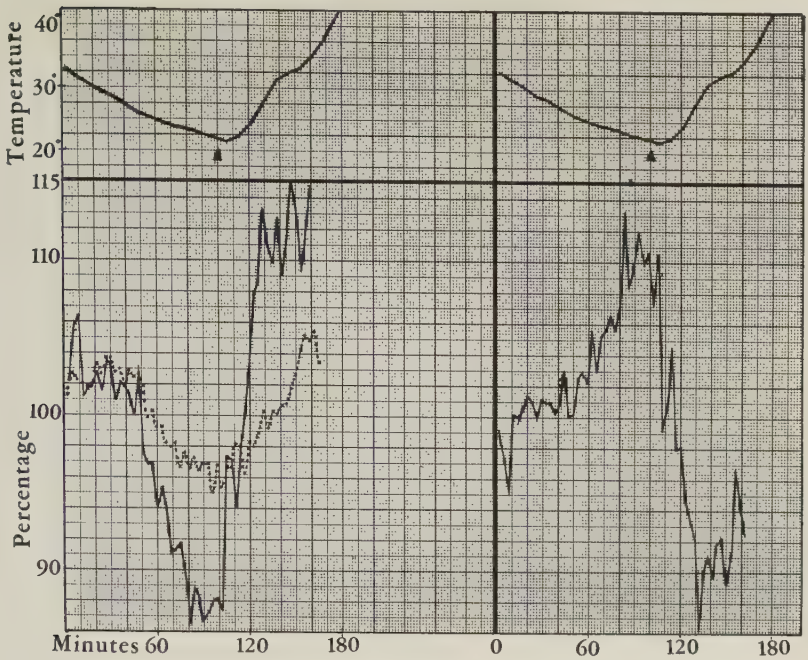

Fig. 4. X-axis, time; each minor division represents three minutes. Y-axis: upper left and right; incubator temperature in degrees Centigrade. Pointer indicates where reheating was commenced. Lower left; solid line—total rate for all 21 cultures expressed as a percentage of the average total rate for the whole episode. Broken line—total potential for all 21 cultures expressed as a percentage of the average total potential for the whole episode. Lower right; potential per beat expressed as a percentage of the average potential per beat for the whole episode.

reheating on the second occasion and % in 30 minutes on the third occasion. e response of the potential per contrac- n to cooling and rewarming seemed to more sensitive than either the rate or ential responses alone and showed the ects of fatigue (in the second experi- nt) as markedly as the others. The anges in the potential per contraction ue with temperature variation, although bably due in large part to changes in rate, carried with them the suggestion t, under the circumstances of the ex- iment, heart tissue had available to it limited amount of energy per unit of he. This amount of expendable energy y have become less with lowered tem- rature of the heart tissue. However, the crease in electrical potential was less n the decrease in heart rate with a ultant increase in the available amount "electrical energy" per beat when tem- rature is lowered. If such a process curs and is exaggerated with further de- crease in temperature, an unstable electrical condition may occur within the heart fibers. A phenomenon such as this may underlie the cardiac arrythmias complicating hypothermia. Further work is in progress on the electrical response of heart tissue to hypothermia.

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The Metabolism of Ejaculated Spermatozoa from the Fowl [1]

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It has been pointed out by a number of investigators, notably Lardy and Phillips ('43) and MacLeod ('41), that studies on the metabolism of ejaculated spermatozoa must be carried out using cells removed from, and washed free of the seminal fluid which normally bathes them. Except for the measurement of endogenous oxygen consumption by Lardy and Phillips ('43), and a more recent report by van Tienhoven ('60), work on cock sperm metabolism has involved the use of whole semen. Bogdoff and Shaffner ('54) reported that the optimum pH for respiration, as measured by methylene blue reduction, of chicken semen was near 7. Zayat and van Tienhoven ('59) presented Z_{O_2} values for cock semen before and after storage for 24 hours at 5°C in three different diluents. Kosin ('44) reported Q_{O_2}, and $Q_{CO_2}{}^{N}$ values, and succinate stimulated respiration of cock semen before and after X-irradiation. Later the oxidative and glycolytic activity of turkey semen was investigated (Kosin, '58), and it was suggested that quantitative aspects of oxidative activity in semen from this animal were similar to those of chicken sperm. Almost nothing is known about the metabolic characteristics of the cock spermatozoan *per se*. Certainly, the biochemical data obtained with these cells suspended in such a complex and undefined medium as seminal plasma are difficult to interpret, and differ considerably, as will be indicated in the present report, when compared with studies on washed cells.

The present investigation is concerned with the respiration and aerobic and anaerobic glycolysis in fowl spermatozoa. In addition, data is presented suggesting breed differences in metabolism which may be of genetic significance.

METHODS

Cock semen was collected[3] by the massage technique of Burrows and Quinn ('37). For most experiments ejaculates from several birds were pooled before use in order to avoid individual variation inherent in the semen from any given animal. The cells were concentrated by gentle centrifugation (700 × G for 15 minutes) and washed two times in a calcium-free Krebs Ringer's phosphate buffered at pH 7.1 (Umbreit et al., '51).

Oxygen consumption of the sperm suspension (3.0 ml final volume) was measured at 37°C in air, using the conventional Warburg apparatus according to the usual procedures (Umbreit et al., '51). The cells were placed in the sidearm of the flask and tipped into the main compartment after a 10–15 minute equilibration period.

Glucose utilization and lactic acid production were used as criteria of glycolytic activity and were determined in $Ba(OH)_2$-$ZnSO_4$ filtrates of the incubates. Anaerobiosis was attained by gassing the flasks for 10 minutes with commercially prepared specially purified (99.99%) nitrogen, while shaking in the constant temperature bath at 37°C, after which, at zero time, the cells were tipped into the main compartments of the flasks.

Glucose was estimated by the Nelson-Somogyi (Somogyi, '52) colorimetric procedure. Lactic acid was determined by the method of Barker and Summerson ('41).

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[3] The authors are indebted to Karl Nestor, Department of Poultry Science, W.V.U., for technical assistance.

In most cases the oxygen consumption of spermatozoal suspensions was expressed in terms of the coefficient Z_{O_2} which represents the cmm O_2 uptake/10^8 cells/hr. at 37°C. Z_G and Z_L represent the µl glucose disappearance and lactate formation respectively per 10^8 cells per hour. Sperm concentrations were determined by counting in a hemocytometer the number of cells in aliquots from the final suspension of washed sperm. At the termination of each experiment the cells were checked for motility, microscopically.

RESULTS

Data from a typical experiment (table 1) reveal that the endogenous O_2 uptake of washed cock sperm declines slightly with time whereas in the presence of glucose there was both stimulation of respiration and maintenance of a constant rate for at least three hours, the longest incubation period used. There was also measurable glucose disappearance and lactate accumulation in the reaction medium. Motility, checked at the end of the incubation, was good. These results are summarized in table 2, which also contains the data on anaerobic glycolysis. From the Z values presented it is apparent that aerobiosis depresses lactate formation, and also that the total amount of glucose utilized anaerobically may be accounted for as lactic acid. On the other hand, in the presence of air, O_2 uptake and lactate accumulation are more than sufficient to balance the glucose disappearance. These data illustrate that a pronounced "Pasteur effect" is present.

Lardy and Phillips ('41) suggested that endogenous phospholipid serves to support respiratory activity of bull sperm. To establish the possible contribution of this potential energy store in cock spermatozoa determinations of phospholipid content of these cells before and after incubation were carried out. An aliquot of the sperm suspension was extracted with an alcohol-ether mixture, evaporated to dryness and the residue wet-ashed with H_2SO_4. The resultant inorganic phosphate was assayed by the colorimetric method of Fiske and Subbarow ('25). The average of 4 determinations was 2.43 µg (2.15–2.71) phospholipid-P/10^8 cells, with no measurable decrease after three hours incubation at 37°C, in the presence or absence of glucose.

That these cells can utilize acetate as substrate, but not as well as glucose, is illustrated by the data in table 3. Further characterization of the respiratory mechanisms of cock sperm is presented in figure 1, which represents an absorption spectrum of a packed suspension of these cells. It was obtained with the spectrophotometer described by Butler and Norris ('60).[4] The absorption spectrum of the sperma-

TABLE 1

Respiration of cock spermatozoa

	O_2 uptake	
	Endogenous	+ Glucose
	µl	µl
First hour	16.9	21.3
Second hour	15.5	20.5
Third hour	15.2	20.5

Incubation medium consisted of Krebs Ringer's phosphate (KRP) at pH 7.1 plus 0.014 M glucose (final concentration) where indicated. T. 37°C. Each flask contained approximately 6.0×10^8 cells.

TABLE 2

Respiration and glycolysis of cock spermatozoa

	Endogenous	Plus glucose	No. of exps.
Z_{O_2}	2.5 ±0.4[1]	3.5±0.2	12
Z_G^{Air}	—	1.4±0.3	5
$Z_G^{N_2}$	—	6.0±0.2	5
Z_L^{Air}	0.27±0.09	1.1±0.2	12
$Z_L^{N_2}$	0.67±0.35	12.2±0.3	5

[1] Figures are the mean and standard error. Incubation medium as in table 1.

TABLE 3

Effect of acetate and glucose on the respiration of cock spermatozoa

Additions	O_2 uptake		
	Exp. 1	Exp. 2	Exp. 3
	µl	µl	µl
None	15.1	31.0	25.4
Acetate	19.2	36.7	31.6
Glucose	24.4	41.1	36.1

Incubation medium as in table 1, plus 0.028 M sodium acetate or 0.014 M glucose where indicated. Incubation time two hours.

[4] Appreciation is expressed to Dr. Warren L. Butler, Department of Plant Industries, U.S.D.A. for his assistance in obtaining these data.

ozoa shows bands due to cytochrome c at 20 and 550 mμ, cytochrome b at 535 and 60 mμ and cytochrome a + a_3 at approxiately 605 mμ. Sodium hydrosulfite was

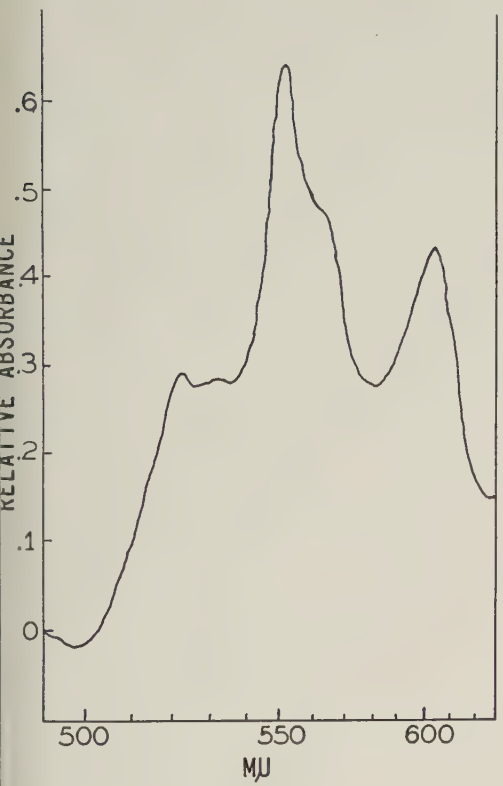

Fig. 1 Absorption spectrum of a packed suspension of cock spermatozoa. Explanation in text.

added to the preparation so that the absorbing components would be in the reduced state. These results indicate that the typical complement of cytochromes is present in these cells.

In table 4 is presented the results of a series of experiments designed to establish whether or not breed differences which exist among these animals may be reflected in the metabolic characteristics of the spermatozoa. The statistical analysis of this O_2 consumption data indicates that significant differences are present. In these experiments ejaculates from 5 to 10 males of a given breed were pooled before washing the cells, and the Z_{O_2} determined as previously described. In addition, the number of live cells in the pooled samples was determined by the differential staining technique of Campbell, Dott and Glover ('56). For any given experiment there was no difference in live cells in samples from the different breeds, nor in the quality of sperm motility. It should be noted that this staining procedure was used routinely during all of this work, to check possible damage to the sperm during washing and we usually obtained from 80–90% live cells, approximately the same as in whole semen.

DISCUSSION

The endogenous Z_{O_2} values reported in these experiments for cock spermatozoa are considerably lower than those obtained by Lardy and Phillips ('43) and by van

TABLE 4

Breed differences in metabolism. Cock spermatozoa

Breed[1]	Z_{O_2}		No. of exps.
	Endogenous	Plus glucose	
RIR	2.5	3.6	7
BPR	3.1	5.0	6
NH	2.5	4.0	7
DW	2.0	3.3	5

[1] RIR, Rhode Island Red; BPR, Barred Plymouth Rock; NH, New Hampshire; DW, Dominant White. Incubation medium as in table 1.

Analysis of variance

Source	d.f.	s.s.	m.s.	F Ratio
Breed	3	13.193	4.398	3.811[2]
Glucose	1	25.347	25.347	21.96[3]
Breed × glucose interaction	3	1.497	0.499	0.432
Error	42	48.45	1.154	

[2] Significant at 5% level.
[3] Significant at 1% level.

Tienhoven ('60), in the few determinations by these authors with washed cells. In comparing the data, it is obvious that, especially with reference to the work of the latter author, a number of differences in experimental methods exist which may account for the differences in our results. In those experiments in which van Tienhoven was concerned with the metabolism of washed sperm rather than whole semen, he measured respiration in Tyrode's solution at pH 6.8 and with fructose as substrate. Also, there was, apparently, dihydrostreptomycin in the medium, and it could conceivably have influenced oxygen uptake by the cells. This author also observed a depression of respiration of whole semen when it was diluted with a phosphate buffer. Whether this would occur with washed cells remains to be determined, although Lardy and Phillips ('43) used Krebs Ringer's phosphate as suspending medium in their determination. Finally, and as suggested by Lardy ('52), methods of handling the animals, frequency of sperm collection and other extraneous factors to which we can add breed of cock, might influence the metabolism of the sperm.

On the other hand, Schindler, Volcani and Weinstein ('58) stated that cock semen lacked, almost completely, glycolytic processes, and van Tienhoven ('60) reported that there was no apparent difference in glycolysis under aerobic and anaerobic conditions. These observations certainly are in complete disagreement with our findings which demonstrate both aerobic and anaerobic degradation of glucose by cock sperm, as well as a "Pasteur effect." And certainly the importance of glycolytic mechanisms for bovine and human sperm is well documented (Mann, '54; MacLeod, '41). The pitfalls inherent in attempting to ascertain the metabolic characteristics of cells in such a complex and undefined medium as seminal plasma, thus become more obvious. Nevertheless, it is interesting to speculate that some component or components of cock seminal plasma may counteract the "Pasteur effect." This requires investigation.

It is of interest to compare these data on respiration and glycolysis of cock sperm with those available in the literature (see Mann, '54, for review) and obtained in our laboratory with bull spermatozoa. Th Meyerhof Oxidation Quotient (MOQ) fo cock sperm in these experiments was 9.5 This calculation is essentially a measur of the metabolic efficiency of the cell, tha is of its hexose sparing ability by efficien energy production and utilization aerob cally. The value obtained here may b compared with Lardy's ('52) report of 9. for bovine epididymal sperm and three t 7 for ejaculated sperm from the bull which sometimes show a "Pasteur effect. According to the latter author, bovine ep didymal sperm have a very low Z_{O_2} as com pared to ejaculated sperm. At least in ou laboratory, the Z_{O_2} of cock sperm is ap proximately ¼ of what we obtain fc ejaculated bull sperm. In other word there appears to be a relatively low oxyge uptake associated with a relatively hig metabolic efficiency as far as these sper matozoa are concerned, with ejaculate cock sperm more economical in terms o energy utilization than ejaculated bul sperm.

In reference to energy production, an in view of the consumption of a measur able quantity of oxygen and the demon stration of the cytochromes in human an bovine sperm (re. Mann, '54), it is no surprising to find the typical complemen of cytochrome pigments present in coc spermatozoa. It is not possible however, o the basis of the data presented in this re port, to state unequivocally that the cyto chrome system serves as the major ter minal oxidative pathway in these cells, nc even that it is functional in respirator metabolism, although this would seem t be a safe assumption. Assays of enzym activity in extracts of these cells are neces sary as supportive data, to establish wit a greater degree of certainty such meta bolic pathways. Indirect evidence of oxida tive phosphorylation which is usually as sociated with cytochrome activity has bee obtained by studying the effect of 2,4-DN on sperm (Norman, Goldberg and Porter field, '60).

The substrate(s) supporting the endog enous respiration and accounting for an aerobic lactate formation in the absenc of glucose has not been identified, thus fa Carbohydrate reserves are notably lackin in spermatozoa (Mann, '54). It has bee

letermined that phospholipid is not serving as an endogenous energy reserve as uggested by Lardy and Phillips ('41) for ull sperm. Recently, we have determined[5] hat glutamic acid is present in relatively igh concentration in cock spermatozoa. urther experiments are in progress to ascertain the role of this compound as a metabolite in these cells.

The most intriguing and most poorly understood finding of this investigation is hat concerned with breed differences in espiration. It is extremely tempting to correlate this result with such things as vigor of sperm motility, or possible differences in fertilizing capacity of the cells; with *in vitro* or *in vivo* longevity, or indeed, even with certain characteristics of the whole animal, both male and female. These birds represent highly inbred groups of animals and one may speculate that these results are of genetic significance. It would be most presumptuous to do so at the present time and on the basis of the very limited data obtained thus far. We can only state that these differences in the oxygen consumption of the sperm have been observed, and suggest that much more extensive study is required to establish the significance of this result.

In summary, we have presented data describing some of the basic biochemical characteristics of avian spermatozoa. These data are of significance from the standpoint of both comparative and reproductive physiology, as well as in providing a base line for future studies in our laboratory, in order to answer some of the questions raised in this report and in the presently available literature, concerning the biochemistry and physiology of both spermatozoa, and cells in general.

SUMMARY

1. Characteristics of the oxidative metabolism of washed cock spermatozoa were determined.

2. Stimulation of respiration of these cells by glucose and acetate was observed, in addition to aerobic and anaerobic glycolysis.

3. Spectrophotometric analysis revealed the presence of the typical complement of cytochromes in the sperm.

4. Significant differences were found in the respiration of spermatozoa obtained from 4 breeds of cocks.

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n Vitro Accumulation of Chlorphenol Red by Rabbit Ciliary Body[1,2]

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Previous studies have shown that organic anions can be used to demonstrate active accumulation in certain tissues; thus, phenol red was reported to be concentrated within the lumina of isolated frog and flounder renal tubules (Foster, 48; Foster and Taggart, '50) and the mesonephros of the chick embryo (Chambers and Kempton, '33). Phenol red was also used to demonstrate the secretory activity of the chorioid plexus of chick, rat, and rabbit embryos and of three-day-old hatched chicks (Cameron, '53). These studies revealed that the process of active dye accumulation against a concentration gradient was one which required the expenditure of energy and that the concentrating ability could be inhibited by cold, anoxia and metabolic inhibitors.

Friedenwald and Stiehler ('38) demonstrated an irreciprocal permeability in the rabbit ciliary body to certain dystuffs. Acid dyes when introduced on the stromal side of the ciliary body filled the stroma and did not penetrate the epithelium, whereas when the dyes were placed on the epithelial side they penetrated into the stroma. The accumulation was blocked by anoxia and cyanide (Friedenwald and Stiehler, '38).

Becker recently demonstrated that the organic anion, iodopyracet (Diodrast) labeled with I^{131}, was accumulated by preparations of rabbit ciliary body and iris *in vitro*. Saturation kinetics could be demonstrated and the concentrating mechanism could be inhibited by metabolic and competitive inhibitors as well as by lowered temperature and the lack of glucose, oxygen or potassium in the incubation media (Becker, '60).

Chlorphenol red was found to be transported in a similar manner to phenol red by the flounder renal tubules (Foster and Hong, '58). The bluish red color of chlorphenol red was more readily perceived and this color was stable within the pH range of tissue maintenance. Because of these advantages over phenol red, this dye was selected for use in the present investigation.

METHOD

Albino rabbits weighing approximately 2.0 kg were sacrificed by air embolism. Their eyes were promptly enucleated and placed in staining dishes containing Tyrode's solution. The eyes were opened posteriorly and the lens and vitreous were dissected free. The ciliary body and iris were removed as a complete ring and placed in another staining dish with Tyrode's solution. The iris was then carefully dissected free leaving the ciliary processes easily visible. The ring of ciliary body was divided into approximately 7 equal pieces. Each piece was placed on a 22-mm cover slip with a drop of Tyrode's solution from a 22-gauge syringe needle. The round cover slip was attached by a drop of Tyrode's solution to a heavier glass slide which was then inverted and placed over Maximow-type culture slides. In this investigation a total of 373 preparations were observed. Incubations were carried out at 27°C and the slides were observed under a microscope at various time intervals. It was found that two hours of incubation was sufficient for maximum accumulation and for a comparison of the

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[3] Student Fellow, National Council to Combat Blindness, Inc.

effects of various inhibitors upon dye accumulation.

The Tyrode's solution contained 0.04 gm% of chlorphenol red and was made up as follows (per liter):

	gm
Sodium chloride	8.00
Potassium chloride	0.20
Calcium chloride	0.20
Magnesium chloride	0.10
Sodium phosphate	0.05
Sodium bicarbonate	1.00
Glucose	1.00

The media were adjusted to pH 7.4–7.5 by bubbling CO_2 through the solution.

RESULTS

Accumulation

After two hours of incubation the dye could be seen within the ciliary processes; the dye uptake could be better seen when the ciliary body pieces were transferred to a dye-free medium. Although in most instances the dye appeared in the stroma, observations were also made of dye concentrated within the capillary lumina of the ciliary processes. There were differences noted in dye uptake in the eyes from different animals; however, specimens from the same animal had about equal dye accumulating capacities.

Requirements in the media

A marked decrease in dye uptake was noted when the media were potassium free or made anoxic by bubbling nitrogen through the media. Glucose free media also caused decrease in dye accumulation. Variations in pH between 7.2 and 8.5 had little effect on this concentrating activity.

Temperature dependence

Dye uptake was negligible when the preparations were incubated at 0°C. When these same preparations were transferred to an incubation temperature of 37°C they promptly showed good dye uptake. Incubation at 37°C showed more rapid dye uptake as compared to controls incubated at 27°C.

Inhibitors

Various known metabolic inhibitors were used to demonstrate the inhibition of dye accumulation. These included dinitrophenol 4×10^{-5} M, sodium cyanide 8×10^{-3} M, and iodoacetate 4×10^{-4} M. Organic acids believed to compete for this transport system were added to the media and these were noted to decrease the dye accumulation when used in the following concentrations: Iodopyracet (Diodrast), 2×10^{-3} M; Acetazolamide (Diamox), 4×10^{-3} M; Penicillin, 1×10^{-3} M; Probenecid (Benemid), 65×10^{-4} M.

DISCUSSION

As in previously studied renal tubules, the rabbit ciliary body has the ability to concentrate chlorphenol red against a concentration gradient. This ability is temperature dependent and requires certain ions and an active metabolic system. This transport system resembles that of the renal tubule as evidenced by the effects of various metabolic and competitive inhibitors. Becker ('60) has estimated the concentration of various inhibitors that results in 50% inhibition of iodopyracet accumulation from linear plots of the reciprocal of the amount accumulated against the concentration of inhibitor in the media. Larger amounts were needed in the present investigation in order to allow the observer to distinguish more clearly differences in dye uptake. The present investigation demonstrates that organic anions such as chlorphenol red are accumulated almost entirely by the ciliary processes. One may therefore estimate that the concentration of iodopyracet in the processes may reach 50–75 times that of the media.

SUMMARY

Pieces of freshly excised rabbit ciliary body accumulate chlorphenol red when incubated in Tyrode's solution. The ability to concentrate the dye is temperature dependent and requires potassium in the media. The concentrating mechanism is inhibited by metabolic inhibitors such as dinitrophenol, iodoacetate and cyanide as well as by related organic anions such as iodopyracet (Diodrast), acetazolamide (Diamox), penicillin and probenecid (Benemid).

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Cytochromes of Marine Invertebrates

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Since many of the marine invertebrates are free from myoglobin and hemoglobin pigments which would interfere with direct spectral measurements of intact cytochromes, they offer great advantages in studying distribution of cytochromes. The cytochromes of some marine invertebrates correspond spectrally to the well known cytochromes $a + a_3$, b, c and c_1 of mammalian mitochondria. Spectral evidence for other cytochromes of the a and b types has been found (Tappel, '60). In our previous study of the cytochromes of muscles of blue crab, American lobster, shrimp, eastern oyster, northern quahog clam, and bay and sea scallops, room temperature difference spectra were used for qualitative and quantitative measurements. More exact qualitative identification was made by spectra taken at liquid nitrogen temperatures. This previous study establishes background for further survey of marine invertebrates because it shows the applicability of direct difference spectra for qualitative assignments of cytochromes and for quantitative measurements.

This paper reports spectral measurements of the cytochromes of a wide variety of invertebrates in the phyla Mollusca and Arthropoda.

MATERIALS AND METHODS

Mollusca, except for squid, scallops, and abalone, were collected alive at low tide along the California Coast. Squid and abalone were obtained refrigerated and scallops were obtained frozen through commercial sources. Arthropoda were collected alive from California Coast except for commercial shrimp and Dungeness crab. Dungeness crab were obtained alive and shrimp refrigerated or frozen from commercial sources. Live animals were maintained as long as possible in refrigerated and aerated sea water and were sacrificed just prior to taking difference spectra. Other specimens were kept either refrigerated at 0°C or frozen. After complete dissection of the animals, samples were selected on the basis of apparent cytochrome content. The samples chosen were mainly heart, liver, kidney and foot muscle of Mollusca and tan colored leg muscles and heart of Arthropoda. The samples were either chopped into fine particles or ground in a tissue press, then homogenized in a glass tube-teflon pestle blendor with 50% gycerol and phosphate buffer 0.1 M pH 7.3. This 50% homogenate is useful because its high viscosity held the sample in suspension and made the preparation translucent. Subcellular particles were prepared from Dungeness crab pancreas by differential centrifugation of a 0.25 M sucrose homogenate. The fraction separating between 2000 × g and 10,000 × g was taken as the mitochondrial fraction.

Difference spectra were taken using the techniques described by Chance, ('54). Identical samples in the oxidized state were used to determine a base line. Then, with one sample reduced and the reference oxidized, difference spectra were recorded with a Beckman DK-2 spectrophotometer, using the sensitive difference scales which record in per cent transmission. Throughout the studies, cytochrome c was used for calibration of the monochrometer. Concentrations were calculated from difference spectra using the wave-length pairs and changes in molar absorbancy indices determined by Chance and Williams, ('56). These are as follows: cytochrome a, 605–630 mμ, 16 $cm^{-1} \times mM^{-1}$; cytochrome b, 564–575 mμ, 20 $cm^{-1} \times mM^{-1}$; cytochrome c, 550–540 mμ, 19

$cm^{-1} \times mM^{-1}$; flavoprotein, 465–510 mμ, 11 $cm^{-1} \times mM^{-1}$; and cytochrome a_3, 445–465 mμ, 91 $cm^{-1} \times mM^{-1}$ for the respiratory pigment, the pair of wavelengths used to measure absorbancy changes, and the changes in molar absorbancy indices, respectively.

RESULTS AND DISCUSSION

Typically good difference spectra are given in figures 1 and 2. The spectra are stacked to include three in each figure. Since the samples vary in cytochrome content, different ranges of per cent transmission are shown for each spectrum. The ordinate gives the scale applicable for each range, and the dashed line gives th position of 100% transmittance. Thes spectra provide details for quantitative in terpretation but are given here mainly a examples to illustrate interpretation c the spectra. Room temperature diffe ence spectra give tentative identificatio of the cytochrome; however, low tem perature spectra would be required fo more positive identification (Estabrook an Mackler, '57). Because the previous stud (Tappel, '60) showed that cytochrome found in marine invertebrates were mainl of the well known types, $a + a_3$, b, $c + c$ it is convenient to show the major result of this research in tables 1 and 2 wher

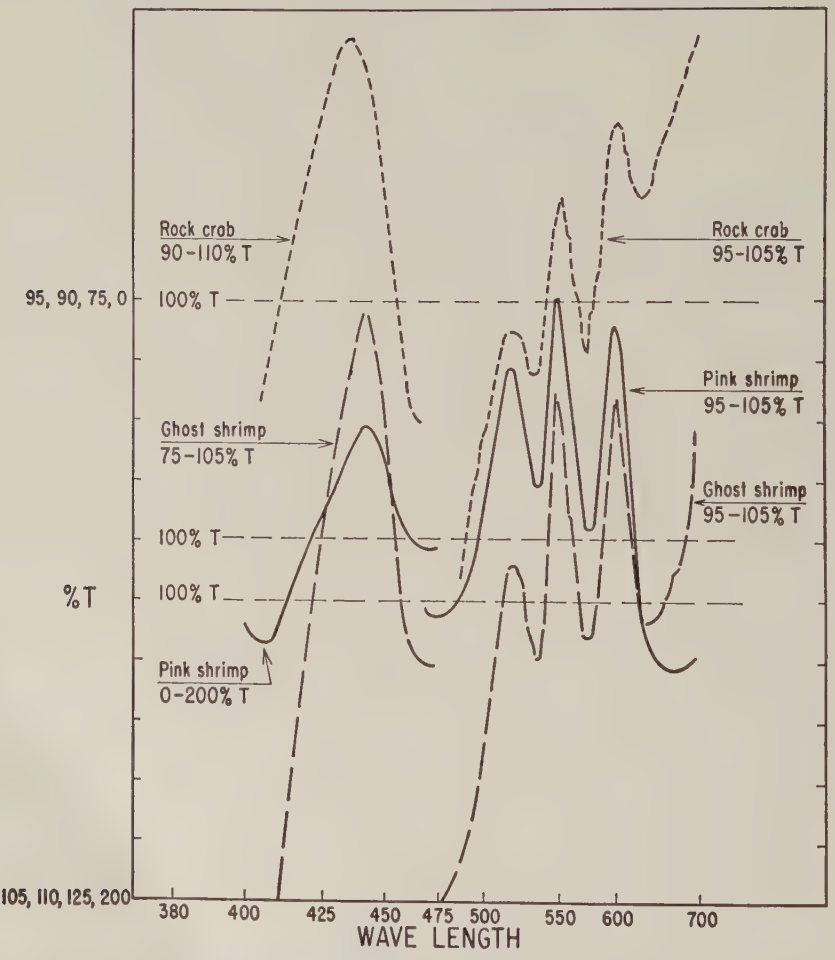

Fig. 1 Difference spectra of cytochrome components of some Arthropoda. Ghost shrimp: pleopod muscle of 60 shrimp, succinate reduced. Pink shrimp: pleopod muscle of 35 shrimp, succinate reduced. Rock crab: muscle of 8 crab, dithionite reduced.

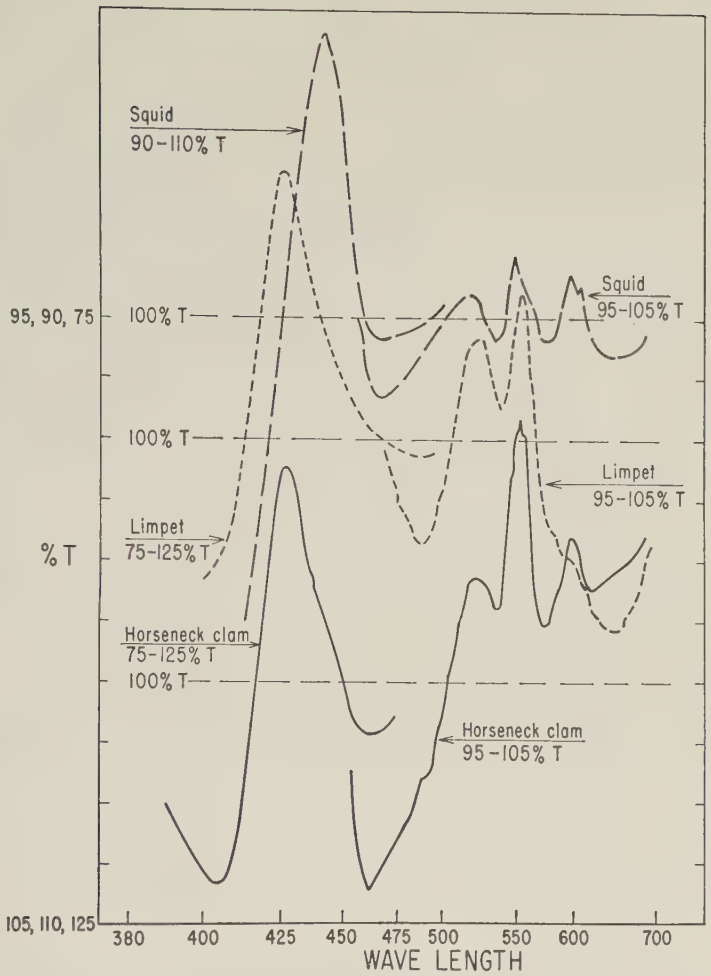

Fig. 2 Difference spectra of cytochrome components of some Mollusca. Horseneck clam: heart muscle of 12 clams, dithionite reduced. Limpet: foot muscle of 15 limpets, dithionite reduced. Squid: heart muscle of 15 squid, succinate reduced.

he spectral characteristics and quantitative data are organized according to these ommon cytochrome types.

Examples in figures 1 and 2 will illustrate the interpretation of spectra. For ock crab the maximum at 607 mμ may be ssigned as the α band of cytochromes of he *a* type. The maxima at 557 mμ may be ssigned as the α band of cytochromes of ne *b* and *c* + c_1 types. The maximum bsorbance at 518–524 mμ corresponds to ne β bands of cytochromes *b* and *c* + c_1 vhile the trough at 468 mμ is used as a ualitative and quantitative measure of avoprotein. In the Soret region maximum absorbance at 439 mμ corresponds to the γ bands for cytochromes of the a_3 and *b* types.

For pink shrimp muscle, the 603 mμ maximum corresponds to cytochrome of the *a* type. The 552 mμ maximum corresponds to combined α bands of cytochrome of the *b* and *c* + c_1 types. The 445 mμ maximum corresponds to the γ peak of cytochrome a_3. The γ peaks of cytochrome *a* + a_3 mask the γ peaks of cytochromes and *b* and *c* due to the large concentration of cytochromes *a* + a_3. Similar interpretations of the spectrum of ghost shrimp muscle allows qualitative identification

TABLE 1

Wavelength (mμ) position of peaks in difference spectra

Organism	Reductant	α			β	Flavo-protein minimum	γ			Cyt. a_3 CO
		Cyt. *a*	Cyt. *b*	Cyt. *c* + c_1	Cyt. *c* + *b*		Cyt. a_3	Cyt. *b*	Cyt. *c*	
PHYLUM MOLLUSCA										
CLASS GASTROPODA										
Haliotis rufescens (Red abalone)										
Foot muscle	Dithionite			–560–		457		430		
Acmaea scabra (Common limpet)										
Foot muscle	Succinate					465		431		
	Pyruvate-malate					465		431		
	Dithionite			–558–	520	466		433		572 α 538 β 421 Max. 436 Min.
Heart muscle	Succinate			–560–		460		–436–		
	β-OH Butyrate			–558–						
	Dithionite			–560–	523	465		432		571 α 563 β 421 Max. 436 Min.
Tegula funebralis (Black tuban shell snail)										
Foot muscle	Dithionite	599		550			444sh.		–427–	416 Max. 445 Min.
	Succinate	600		552		465	443sh.	432		
	β-OH Butyrate	604		552		467		435		
Liver	Dithionite		562			467	444sh.		–427–	416 Max. 431 Min.
	Succinate							431		
	β-OH Butyrate			–559–				–437–		
Polinices lewisii (Moon snail)										
Kidney	Dithionite			–558–	530		445sh.		–427–	
Heart muscle	Dithionite	606		–556–	516	465		432		
Liver	Dithionite	600		–558–	524			431		
CLASS PELECYPODA (LAMELLIBRANCHIATA)										
Macoma secta (White sand clam)										
Foot muscle	Dithionite	602		–554–	521	468		428		

	Succinate		–560–		531	460		429	
	β-OH Butyrate		–560–					428	
Heart muscle	Dithionite	604	–558–		526	465	445sh.	430	
	Succinate	605	–554–		524			430	
	β-OH Butyrate	605	–553–					431	
Stomach muscle	Dithionite	608	–560–		532	465	447sh.	428	
	Succinate		–558–					430	
			–558–					430	
Foot muscle	Dithionite	605	–556–		528	465		430	421 Max. 440 Min.
	β-OH Butyrate	602	–561–			464		–427–	
Mytilus edulis (Bay mussel)									
Mantel	Dithionite		–559–			462	440sh.	429	
Pecten magellanicus (Sea scallop)									
Foot muscle	Dithionite	608		551	521	464		434	
	Succinate	605	–554–		521	465	447sh.	428	
CLASS CEPHALOPODA									
Loligo opalescens (Squid)									
Heart	Dithionite	602	–556–		520	469	443sh.	433	590 α 548 β 426 Max. 446 Min.
Heart muscle	β-OH Butyrate	602		551	521	470	446	436sh.	
	Succinate	602	562sh.	551	519	467	445sh.	434	
PHYLUM ARTHROPODA									
CLASS CRUSTACEA									
Cancer antennarius (Rock crab)									
Muscle	Dithionite	604		552	520	469		428	
	Succinate	604	–554–						
Heart muscle	Dithionite	601	563					430	
	Succinate		–554–					431	
Gills	Dithionite		–561–			466	444sh.	429	
Hemigrapsus nudus (Purple shore crab)									
Heart muscle	Dithionite	604	–554–			466	–440–		
Pachygrapsus crassipes (Striped shore crab)									
Heart muscle	Dithionite		564					430	
Gills	Dithionite	608	–561–			465		432	
	Succinate	607	–554–		521		441sh.	–427–	
Cancer magister (Dungeness crab)									
Heart muscle	Dithionite	601	–557–		523			430	
Tan muscle	Dithionite	600	–560–		514	466	443sh.	433	
Particles	Dithionite	600	–558–			465	443	443sh.	
Pancreas	Dithionite	607	–557–		526	467	443sh.	432	

TABLE 1 (*Continued*)

Wavelength (mμ) position of peaks in difference spectra

Organism	Reductant	α			β	Flavo-protein minimum	γ			Cyt. a_3 CO
		Cyt. *a*	Cyt. *b*	Cyt. $c + c_1$	Cyt. $c + b$		Cyt. a_3	Cyt. *b*	Cyt. *c*	
Callianassa californiensis (Ghost shrimp)										
Pleopod muscle	Dithionite	604		551	521	465	442			
	Succinate	604	562sh.	551	521	462	445			574 α 537 β 428 Max. 446 Min.
Pandalus jordani (Pink shrimp)										
Pleopod muscle	β-OH Butyrate	604	562sh.	551	520	476	445			588 α 550 β 430 Max. 446 Min.
Pleopod muscle	Dithionite	604	561	551	522	470	444			
	Succinate	600	560sh.	551	520	468	444			589 α 550 β 429 Max. 446 Min.
Heart muscle	Dithionite	604	562sh.	552	522	470	445			590 α 547 β 427 Max. 446 Min.
	Succinate	604	560	553	524	470	445			
	β-OH Butyrate	602		550	520	470	445			
	Pyruvate-malate	601		550	520	469	445			
Pandalus platyceros (Prawn or spot)										
Pleopod muscle	Dithionite	602	558sh.	550	521	464	444			587 α 547 β 415 Max. 445 Min.
	Succinate	602	560sh.	550	524	467	445			
Penaeus aztecus (Brown jumbo-sized prawn)										
Pleopod muscle	Dithionite	602	–558–		524	464	442			586 α 538 β 426 Max. 445 Min.
	Succinate	602	560sh.	550	520	467	444			
	β-OH Butyrate	602		551	520	471	445			
Penaeus setiferus (White medium sized shrimp)										
Pleopod muscle	Dithionite	605	560		521	460		435		

nd quantitative measurements of cyto-
romes of the $a + a_3$, b and $c + c_1$ type.
Cytochrome patterns of some mollusca
re given in figure 2 as typical examples.
n the limpet foot muscle spectra there is
o evidence for the occurrence of cyto-
hrome of the $a + a_3$ type in the α region.
his is probably due to a very low concen-
ation of cytochromes of the $a + a_3$ type.
he sharp maximum absorbance at 560
μ is a qualitative and quantitative meas-
re of cytochromes of the b and $c + c_1$
pes. The maximum at 429 mμ probably
rresponds to the γ band of cytochrome b.
ue to the relatively low concentration of
tochrome a_3, the γ band of cytochrome
, must be obscured by the γ bands of
tochromes b and c.

The difference spectra of horseneck
am heart indicates the occurrence of
tochromes of the $a + a_3$, b and $c + c_1$
pes. The maximum at 604 mμ corre-
onds to cytochromes of the $a + a_3$ type.
he absorbance maximum at 558 mμ cor-
sponds to cytochromes of the b and c
pes. The 430 mμ maximum in the Soret
gion corresponds to that of cytochrome

A similar interpretation can be applied
the difference spectrum of heart muscle
squid which shows a maximum at 600
μ corresponding to cytochromes of the
$+ a_3$ type and maximum at 552 mμ cor-
sponding to cytochromes of the b and c
pes. The maximum at 444 mμ corre-
onds to the γ band of cytochrome a_3.

Since there has been no previous re-
arch on the cytochromes of the marine
vertebrates reported here (Gumbmann
al., '58; Tappel, '60), the total cyto-
romes are first qualitatively and quanti-
vely measured using sodium dithionite
a reductant. For samples which gave
good difference spectrum with dithionite
duction, substrate reduction using succi-
te or β-hydroxybutyrate was made. In
any cases we failed to get good differ-
ce spectra with enzymic reduction.
hile chemical reduction reduces all of
e cytochromes present, enzymic reduc-
n usually gave only partial reduction.
ability to get enzymic reduction probably
rrelates with inactivation of the cyto-
rome electron transport system during
frigerated and frozen storage and prepa-
ration of the sample. The use of 50% glycerol would probably account for some inactivation of the cytochrome electron transport system.

Table 1 summarizes the absorbance characteristics of difference spectra found for a wide variety of Mollusca and Arthropoda. These have been classified according to the absorbance characteristics of the well known cytochromes $a + a_3$, b and $c + c_1$ with which they may be tentatively identified.

Carbon monoxide difference spectra are very useful in classifying cytochromes of the a_3 type (Chance, '53; Chance and Williams, '56). Arthropoda cytochromes, studied here and in previous work, give carbon monoxide difference spectra essentially identical to that of cytochrome a_3. The carbon monoxide spectra of the Mollusca indicates that they have a cytochrome of the a type which is characteristic of Mollusca but different from the well known cytochrome a_3 of animals. This cytochrome gives carbon monoxide difference spectra with the following characteristics: α, 570–572 mμ; β, 536–540 mμ; γ maxima, 412–421 mμ; and γ minima, 433–445 mμ. The data in table 1 on carbon monoxide difference spectra and those in the previous study show that this cytochrome is widely distributed in the Mollusca. Its further characterization would be very interesting. In reduced-oxidized difference spectra, limpets and mussels gave no absorbance bands indicating cytochromes of the $a + a_3$ type. However, carbon monoxide difference spectra of limpet foot muscle and heart muscle gave good spectral evidence of cytochromes of the a type. Squid is a notable exception among the Mollusca because it has a carbon monoxide difference spectra like the Arthropoda and indicative that it contains cytochrome a_3.

The concentrations of cytochromes given in table 2 are necessarily approximations. They are useful in identifying the tissues containing the largest concentrations (shrimp pleopod muscle) and in giving generalities of cytochrome patterns in the terminal electron transport chain of marine invertebrates. The concentrations of cytochromes are low in Mollusca and there

TABLE 2

Concentrations of the respiratory pigments of marine invertebrates

Organism and sample size	Reductant	Respiratory pigments μmoles/kg wet weight				
		Cyt. *a*	Cyt. a_3	Cyt. *b*	Cyt. $c + c_1$	Flavo-protein
PHYLUM MOLLUSCA						
CLASS GASTROPODA						
Haliotis rufescens (Red abalone)						
Foot muscle (1)	Dithionite			1.7		1.5
Acmaea scabra (Common limpet)						
Foot muscle (10)	Dithionite		0.23	1.8	1.2	
Acmaea (sp) (Limpet)						
Foot muscle (15)	Dithionite		2.4	3.0	2.7	
Tegula funebralis (Black tuban shell snail)						
Foot muscle (10)	Dithionite	1.9	3.8		2.0	
Liver (10)	Dithionite		2.6	2.0		
Polinices lewisii (Moon snail)						
Kidney (1)	Dithionite		3.3	3.2	3.2	
Heart muscle (1)	Dithionite	3.2	13.6	8.6	11.2	24.0
Liver (1)	Dithionite	1.4	9.7	18.2	9.4	
CLASS PELECYPODA (LAMELLIBRANCHIATA)						
Macoma secta (White sand clam)						
Foot muscle (22)	Dithionite		1.8	2.3	3.3	4.8
Schizothaerus nuttallii (Horseneck clam, gaper, rubberneck clam)						
Liver (4)	Succinate		0.5	1.6		
	β-OH Butyrate		1.1	2.6		
Heart muscle (12)	Dithionite	1.3	1.7	3.0	2.4	6.5
Stomach muscle (4)	Dithionite		1.2	2.9	2.0	
Foot muscle (3)	Dithionite	0.3	1.3	2.5	1.8	7.0
Pecten magellanicus (Sea scallop)						
Foot muscle (4)	Dithionite	1.3	0.8	0.6	2.5	4.6
	Succinate	1.0	1.3	1.2	2.2	3.0

CLASS CEPHALOPODA						
Loligo opalescens						
(Squid)						
Heart (6)	Dithionite	3.1	5.0	2.6	1.2	
Heart muscle (15)	β-OH Butyrate	2.0	1.7		2.0	
	Succinate	1.9	2.5	0.9	1.4	3.0
PHYLUM ARTHROPODA						
CLASS CRUSTACEA						
Cancer antennarius						
(Rock crab)						
Tan muscle	Dithionite	1.5	1.5	0.4	1.4	
Heart muscle (8)	Dithionite	1.3	0.3	1.9		
Hemigrapus nudus						
(Purple shore crab)						
Heart muscle (60)	Dithionite	2.0	4.7	0.8		
Pachygraspus crassipes						
(Striped shore crab)						
Gills (40)	Dithionite	0.6	0.5	1.6		1.5
	Succinate	1.1	1.4	1.2	1.7	
Cancer magister						
(Dungeness crab)						
Tan muscle (1)	Dithionite	0.6	3.3	4.0	1.3	12.7
Pancreas (3)	Dithionite	2.2	6.5	6.0	3.4	
Particles (4)	Dithionite	3.7	4.0	0.2	0.3	
Callianassa californiensis						
(Ghost shrimp)						
Pleopod muscle (60)	Dithionite	6.9	8.4	2.9	7.1	
	Succinate	5.3	7.7	1.9	5.0	
Pandalus jordani						
(Pink shrimp)						
Pleopod muscle (35)	Dithionite	8.6	8.3	3.3	6.6	11.8
	β-OH Butyrate	5.8	11.0	1.3	3.9	
	Succinate	7.5	11.5	1.9	3.6	
Heart muscle (60)	Dithionite	3.6	4.2	1.9	1.7	5.7
	β-OH Butyrate	4.1	5.4	1.1	0.2	3.6
	Pyruvate malate	4.5	5.1		2.1	1.8
Pandalus platyceros						
(Prawn or spot)						
Pleopod muscle (3)	Dithionite	18.7	21.2	7.2	12.7	
	Succinate	15.9	16.1	2.8	10.8	13.9
Penaeus aztecus						
Pleopod muscle (3)	Dithionite	13.3	13.8	5.3	9.5	18.3
	Succinate	9.8	11.8	2.9	6.9	
	β-OH Butyrate	10.7	13.5	0.9	8.6	15.1
Penaeus setiferus						
Pleopod muscle (12)	Succinate	14.1	9.1	3.5	11.1	
	β-OH Butyrate	9.4	15.0	1.6	8.4	

are generally similar amounts of cytochromes of the *a*, *b* and *c* types. In Arthropoda, shrimp are noteworthy in having relatively high concentrations of cytochromes occurring in the pleopod muscle and in having cytochromes $a + a_3$ in higher concentrations than cytochromes *b* and $c + c_1$.

SUMMARY

Cytochrome respiratory pigments of a wide selection of Mollusca and Arthropoda have been measured by their characteristic spectral absorbance. Muscles and organs of these marine invertebrates gave difference spectra similar to those of the mitochondria of mammals; the cytochromes are predominately of the $a + a_3$, *b* and $c + c_1$ types. Carbon monoxide spectra of Mollusca indicate a characteristic cytochrome of the *a* type different from a_3. The highest cytochrome concentrations, 10–20 μmoles cytochrome *a* and a_3/kg wet weight, were found in shrimp pleopod muscles.

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ne Cellulolytic Activity in the Snail *Levantina ierosolyma* Boiss

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Helix pomatia is considered one of the w metazoons capable of self-production cellulase (Biederman and Moritz, 1898; rrer, Schubert and Wehrli, '25; Karrer d Schubert, '26). It is presumed that nile homogenates of the salivary gland, e crop-stomach and the hepatopancreas e capable of decomposing Na carboxy-ethyl cellulose, cellulose *per se* is di-sted only by homogenates of the stom-h and the hepatopancreas (Myers and orthcote, '58; Stone and Morton, '58).

On the other hand Florkin and Lozet 49) have demonstrated the presence of llulose-decomposing bacteria in the di-stive tract of *H. pomatia*, and maintain at it is these bacteria that are respon-ble for breaking down cellulose in the ail's intestine.

Myers and Northcote ('59) write: "It is ggested from the present work, that nce a number of cellulases occur in the gestive tract of *H. pomatia*, it is possible at one or more of these are produced by e intestinal flora and others by the ani-al itself."

The purpose of the present study was e investigation of the source of the en-mes that decompose cellulose in the ali-entary canal of *Levantina hierosolyma*. his was accomplished by comparing the llulolytic activity of normal snails with at of snails whose digestive tract had en sterilized with antibiotics, and also rough comparison of cellulolytic activ-y in the various parts of the alimentary nal of both active and estivating snails.

MATERIALS AND METHODS

Estivating snails were collected in the cinity of Jerusalem during July and ugust and were kept in 10 × 15 × 20 cm lyethylene boxes, lined with blotting per. A number of snails were induced to activity by maintaining in a damp environment, and were kept active for at least one month before being dissected.

Sterilization of the alimentary canal. The shells of the snails were cleaned with 70% ethyl alcohol and the animals were then placed in deep, sterile Petri dishes. They were given a mixture of sterile flour and antibiotics (1:1) as well as sterile distilled water. Among the antibiotics used were aureomycin, penicillin together with streptomycin and achromycin; mycostatin was employed as an antifungal measure. The snails were transferred every 24 hours to fresh Petri dishes. Dissection of snails was carried out only after their feces were proven sterile by culture test. In order to confirm sterility of the intestine during dissection, portions of the alimentary tract were cultured on the following media: peptone-yeast extract infusion, peptone-yeast extract agar, brain heart infusion, cellulose infusion, cellulose-agar and soil extract infusion.

Preparation of extract. After rinsing its shell in 70% alcohol the snail was dissected under aseptic conditions. At first the shell was removed. An incision was then made along the dorsal surface, following which the alimentary tract was excised and salivary gland, crop-stomach and hepatopancreas separated.

Salivary gland. Depending on the nature of the experiment the salivary gland was either washed or not prior to homogenation, and then weighed to an accuracy of 0.1 mg. Homogenation was carried out, at room temperature with distilled water at 1:10, and was followed by centrifugation for 20 minutes at 6000 rpm, the supernatant serving as the enzyme solution.

Crop-stomach. These organs were dissected along their length and washed with sterile, distilled water in order to remove

all contents. They were then treated in the same manner as described for the salivary gland.

Hepatopancreas. Parts of the intestine passing through the hepatopancreas were removed, remaining portions being processed as above.

Determination of enzymatic activity. Enzymatic activity was estimated from the rate of decomposition of Na carboxymethyl cellulose (CMC). Degradation of Na-CMC by cellulase diminishes the viscosity of the solution, the decrease in viscosity being directly proportional to the rate of degradation. The extent of activity of the enzyme was therefore determined by the decrease in the rate of flow (ROF) of a 1% solution of Na-CMC of Cekol U.V. type, the latter kindly supplied by Uddeholms Actibolag of Sweden. Viscosity was measured by means of an Oswald viscometer maintained in a water bath at a constant temperature of 36.7 ± 0.1°C. The viscometer was filled with 5cc of a 1% solution of Na-CMC and 2 cm³ of a citrate buffer solution. After the temperature of the mixture equaled that of the water bath, the initial ROF was measured with a stop watch; 0.1 cm³ enzyme extract was then added, and the ensuing change in ROF was calculated in per cent of the initial reading.

Buffer solution. Citrate buffer of pH 5.6 was prepared to an accuracy of 0.1 units. In order to determine the optimal pH, citrate buffers and phosphate buffers within a pH range of 3.5–8.0, were tried out.

Michaelis constant. The Km was determined by measuring the viscosity of a solution of Batch S 121 CMC with a molecular weight of 15,000–30,000. Various percentage concentrations of CMC were used, the reaction solution always containing 5 cm³ CMC + 2 cm³ buffer + 0.1 cm³ enzyme solution.

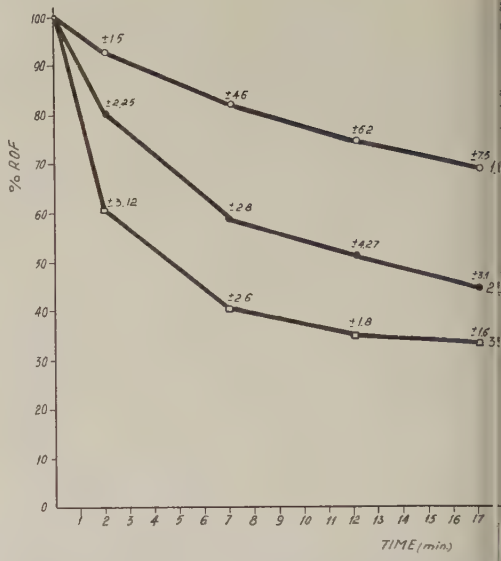

Fig. 1 ROF, in per cent, measured following the addition of 0.1 cm³ of an enzyme solution obtained from various parts of the alimentary tract 1, Salivary gland; 2, crop-stomach; 3, hepatopancreas. Each point in the graph represents an average of 8 determinations carried out on 8 different snails. The standard error for each point is given.

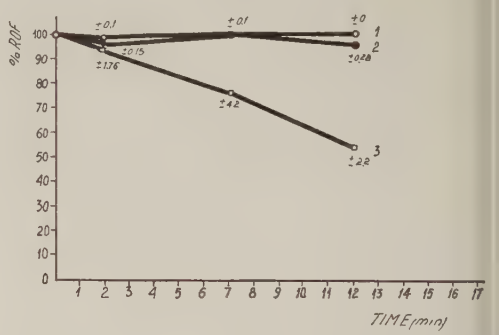

Fig. 2 ROF, in per cent, measured following the addition of 0.1 cm³ of an enzyme solution obtained from various parts of the alimentary tract after washing. 1, Salivary gland; 2, crop-stomach; 3, hepatopancreas. Each point in the graph represents an average of 6 determinations. The standard error is given.

RESULTS

Experiment A. Determinations of cellulolytic activity in active snails not treated with antibiotics (fig. 1) showed decomposition of CMC by extracts of the salivary gland, crop-stomach, and hepatopancreas. The greatest activity was recorded for the hepatopancreas, the weakest for the salivary gland.

Experiment B. An experiment similar to the previous one was carried out with untreated active snails. In this instance however, the organs were thoroughly rinsed prior to homogenation so as to ascertain whether the enzyme was present

the lumen of the particular organ or as intra-cellular. It was found that cellulolytic activity in the salivary gland as well as in crop-stomach ceased following rinsing of these organs, but persisted in the hepatopancreas (fig. 2).

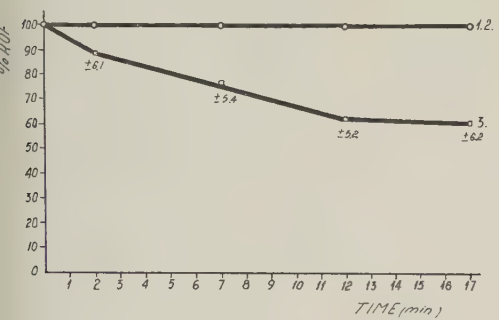

Fig. 3 ROF, in per cent, measured following the addition of 0.1 cm³ of an enzyme solution obtained from various parts of the alimentary tract of estivating snails. 1, Salivary gland; 2, crop-stomach; 3, hepatopancreas. Each point in the graph represents an average of 6 determinations carried out on 6 different snails. The standard error is given for the hepatopancreas. As regards the salivary gland and crop-stomach, no standard error is given, since in all instances the error did not exceed ± 0.1.

Experiment C. Determinations of the cellulolytic activity of estivating snails offered surprising results: On the one hand there was complete cessation of cellulolytic activity in the salivary gland as well as in crop-stomach, but, on the other hand, there was persistent though slightly reduced activity in the hepatopancreas (fig. 3).

Experiment D. In the case of estivating snails, too, the cellulolytic activity of the hepatopancreas persisted even after thorough washing though at a slightly reduced rate[1] (fig. 4).

Experiment E. Determination of cellulolytic activity in active snails following treatment with antibiotics showed cessation of activity in the salivary gland as well as in the crop-stomach, but persistent activity in the hepatopancreas (fig. 5). In cases where treatment with antibiotics failed to sterilize the alimentry tract, cellulolytic activity persisted in the salivary gland as well as in the crop-stomach.

[1] Since washing was carried out with distilled water, hydrolysis of cells might possibly have occurred, thus accounting for the decrease in activity.

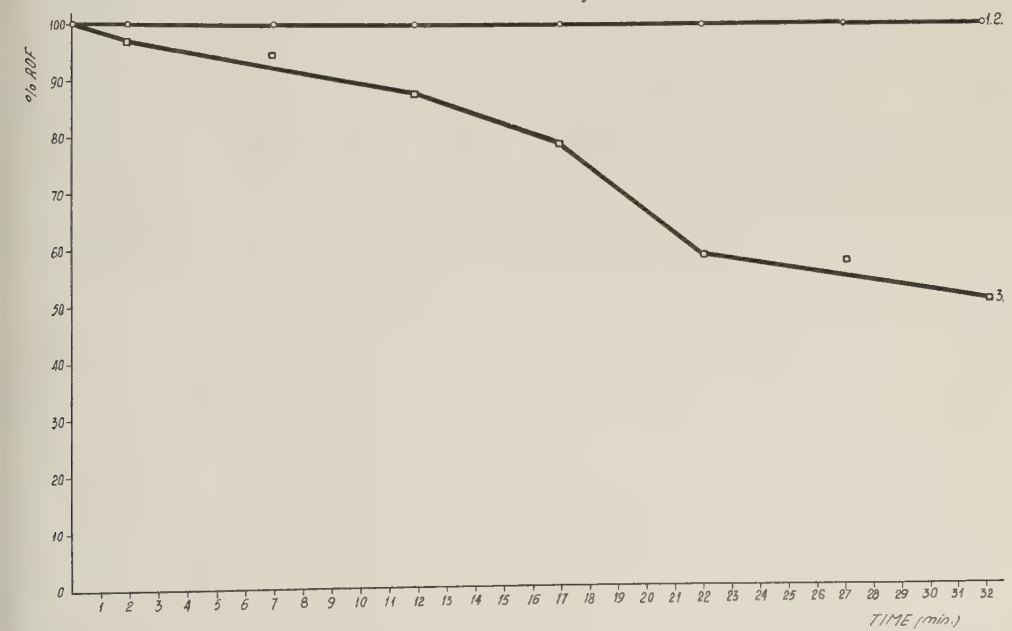

Fig. 4 ROF, in per cent, measured following the addition of 0.1 cm³ of an enzyme solution obtained from various parts of the alimentary tract of estivating snails after washing of the organs. 1, Salivary gland; 2, crop-stomach; 3, hepatopancreas. The graph represents an average of 4 experiments.

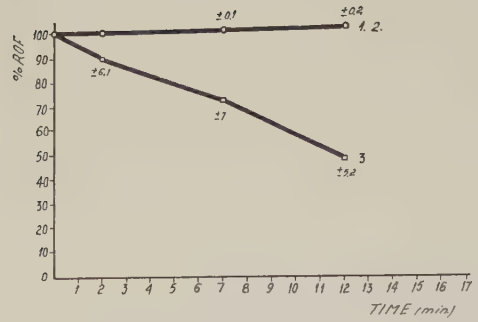

Fig. 5 ROF, in per cent, measured following the addition of 0.1 cm³ of an enzyme solution obtained from various parts of the alimentary tract of snails subsequent to treatment with antibiotics. 1, Salivary gland; 2, crop-stomach; 3, hepatopancreas. Each point in the graph represents an average of 10 experiments carried out on 10 different snails. The standard error is given.

Experiment F. Parts of the crop-stomach and hepatopancreas of an untreated, active snail were placed in separate test tubes containing nutrient medium and cellulose infusion at 30°C, and were tested for celluloyltic activity by the end of 24 hours (table 1).

A second experiment was carried out under similar conditions, but this time using parts of the alimentary tract obtained from a "sterile" snail. No cellulolytic activity was observed in the test tube containing the crop-stomach whereas the one containing the hepatopancreas showed

TABLE 1

ROF in seconds after 24-hour incubation of parts of alimentary canal of normal snail

ROF determination time	Crop-stomach	Hepato-pancreas
min.		
0	42.0	42.0
2	38.0	35.0
7	29.2	22.2
12	27.8	18.0

TABLE 2

ROF in seconds after 24-hour incubation of parts of alimentary canal of "sterile" snail

ROF determination time	Crop-stomach	Hepato-pancreas
min.		
0	41.2	41.0
2	41.2	36.2
7	40.8	26.2
12	40.8	21.8

marked activity (table 2). This suggests that the cellulolytic activity may indeed be attributed to the enzyme extract and not to bacterial secretions, particularly since none of the test tubes showed development of bacteria.

Experiment G. Further tests were carried out in order to verify the fact that cellulolytic activity was indeed instigated by an enzyme dissolved out of the tissues. Sections of crop-stomach and hepatopan-

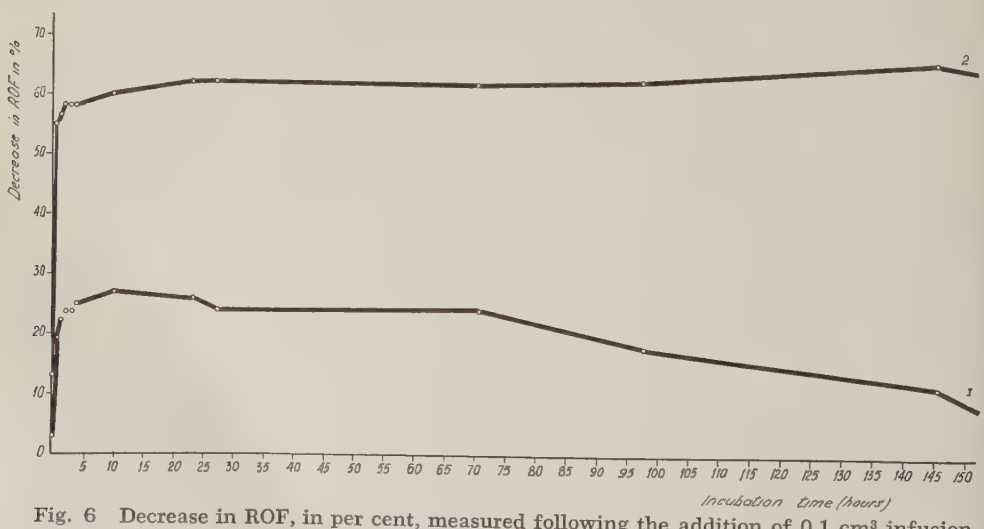

Fig. 6 Decrease in ROF, in per cent, measured following the addition of 0.1 cm³ infusion enzyme extract. 1, Crop-stomach; 2, hepatopancreas.

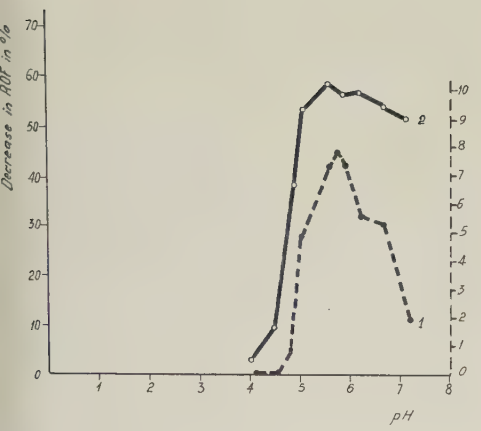

Fig. 7 Decrease in ROF, in per cent, measured following the addition of 0.1 cm³ enzyme solution obtained from various parts of alimentary tract. 1, Crop-stomach; 2, hepatopancreas.

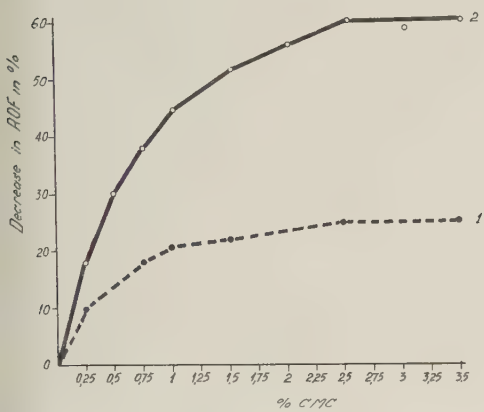

Fig. 8 Decrease in ROF, in per cent, measured following the addition of 0.1 cm³ of an enzyme solution obtained from various parts of the alimentary tract. 1, Crop-stomach; 2, hepatopancreas.

reas were placed in test tubes, and tested t half-hour intervals for cellulolytic activity. It was assumed that if the enzymes ere after all of bacterial origin, the rise cellulolytic activity should correspond a bacterial growth curve. Results of ese tests (fig. 6) showed that for sections of hepatopancreas, cellulolytic activity reached a maximum two hours llowing inoculation, persisted at a maximal level for about 200 hours, and subsequently decreased gradually. There was similar rise in the case of the crop-omach, but here a decrease in cellulotic activity occurred already by the end of 70 hours. It was further noted that bacterial growth was far more intense in the test tube containing crop-stomach sections.

Experiment H. Optimal pH levels for enzyme activity were determined both for crop-stomach and for hepatopancreas sections. A pH of 5.6 was found to be optimal for extract of hepatopancreas acting on Na-CMC, whereas for the crop-stomach the optimal pH was 5.75 (fig. 7).

Experiment I. The Michaelis constant was determined for the enzyme in crude extracts of the hepatopancreas as well as the crop-stomach. This was done by reacting the extract with Batch S 121 Na-CMC of a molecular weight of 15,000–30,000. For the crop-stomach and the hepatopancreas these constants were 1.5–2.5×10^{-4} M, and 1.7–3.1×10^{-4} M respectively (fig. 8).

DISCUSSION

In the course of our experiments we found cellulolytic activity in the unwashed salivary gland, crop-stomach, and hepatopancreas of *Levantina hierosolyma.* Activity in the salivary gland and crop-stomach ceased after washing, but persisted in the hepatopancreas (experiments A and B). These observations indicate that the cellulases found in the salivary gland and in the crop-stomach are not products of these organs. The presence of cellulases in these organs may be attributable either to passage of these enzymes from the hepatopancreas, where they are apparently secreted, or to a local bacterial secretion as reported by Florkin and Lozet ('49) for *H. pomatia.*

Noteworthy is the fact that cellulolytic activity both in the salivary gland and in the crop-stomach ceases during estivation of the snail (experiment C). This may be explained in two ways: either passage of enzymes from the hepatopancreas to the salivary gland and the crop-stomach is interrupted during estivation, or else the state of estivation, during which the snail ceases feeding, is unfavorable for the development of cellulose-decomposing bacteria. In support of the first possibility there are the investigations of Eckstein and Abraham ('59) that suggest reduced

general metabolic activity of the hepatopancreas during estivation, as evidenced by a decrease in the activity of dehydrogenases. The latter possibility is supported by the findings of Galli and Giese ('59) who noted a decrease in the number of bacteria in the intestine of *Tegula* kept in the laboratory without any special treatment.

In our experiments with snails whose alimentary tracts had been freed of bacteria, cellulolytic activity persisted only in homogenates of the hepatopancreas. This clearly indicates that cellulases found in the hepatopancreas are secreted by the organ itself, whereas, on the other hand, there is no evidence of production of cellulases by either the salivary gland or the crop-stomach.

The above observation tends to contradict the viewpoint that holds that the cellulose-decomposing enzymes are transferred from the hepatopancreas to the salivary gland and the crop-stomach; were the latter true then cellulolytic activity in the case of "sterile" snails should have been found in these regions as well. Our observations, in fact, favor the assumption of a bacterial origin for the cellulases in the salivary gland and in the crop-stomach.

In experiments G and F parts of the alimentary tract of both sterile and unsterile snails were placed in test tubes containing nutrient culture media and subsequently tested for cellulolytic activity. No activity was observed in the test tube containing parts of the crop-stomach of a "sterilized" snail, whereas activity was reported from the test tube containing portions of hepatopancreas. The absence of any bacterial growth in both test tubes proves that the enzyme could not have been the result of bacterial secretion.

Additional proof is derived from experiment H in which the rate of change of activity was investigated and failed to show any correspondence to a bacterial growth curve. Such peak of cellulolytic activity as was reached within two hours suggests the diffusion of an existing enzyme rather than the production of an enzyme by developing bacteria. In the case of the crop-stomach, the rapid drop in activity within 70 hours, as opposed to the 200 hours required for such drop by the hepatopancreas, suggests a rich bacterial flora in the former organ that apparently destroys the enzymes. Destruction of the enzymes in the hepatopancreas is a slower process since normally this organ contains far fewer bacteria.

In their investigations Myers and Northcote ('59) pointed to the existence of a number of cellulose-decomposing enzymes. They further found the optimal pH for crude extract reacted with cellofase B to be 5.6. In the present study, however, a comparison of optimal pH for cellulolytic activity in both the hepatopancreas and crop-stomach shows no significant difference in this respect between the two organs, the optimal pH for the former being 5.6 and for the latter, 5.75. This slight difference does not warrant postulating the existence of two separate enzymes. Furthermore, neither are there any appreciable differences between the Michaelis constants for the organs under discussion and hence it would seem that there are no sufficient grounds for postulating the existence of two separate enzymes. The latter results, however, may be due to the use of unpurified extracts.

Finally, it may be concluded from the above-described series of experiments, that the decomposition of cellulose in the hepatopancreas of *Levantina hierosolyma* is accomplished through the secretion of cellulolytic enzymes by this tissue. On the other hand, decomposition of cellulose in the salivary gland in the crop-stomach may be attributed either to bacterial activity or to the passage of cellulolytic enzymes from the hepatopancreas.

SUMMARY

1. Cellulolytic activity has been determined in the salivary gland, crop-stomach and the hepatopancreas.

2. Thorough washing of the digestive tract prior to homogenation resulted in the complete cessation of cellulolytic activity in the salivary gland and the crop-stomach. In the hepatopancreas, however, enzymatic activity persisted, though at a somewhat lower level.

3. In estivating snails, there is no cellulolytic activity in the salivary gland or

op-stomach but such activity persists in e hepatopancreas.

4. Snails that have undergone treatent with antibiotics showed no cellulolic activity in the salivary gland or op-stomach but persistent activity in the patopancreas.

5. Cellulolytic activity was established tubes containing liquid bacterial culre media in which either portions of the op-stomach or hepatopancreas of normal ails had been placed. When the produre was repeated for snails treated th antibiotics, cellulolytic activity was served only in the tube containing hepopancreas.

6. Cellulolytic activity was determined so in tubes examined at half-hour interls. The rise in activity was not found correspond to a bacterial growth curve.

7. The optimal pH for cellulolytic acvity in the crop-stomach was found to be 75. The corresponding pH for hepatoncreas was 5.6.

8. Michaelis constants for the cropomach and hepatopancreas were estabshed as $1.5–2.5 \times 10^{-4}$ M and 1.7–3.0 10^{-4} M respectively.

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